REMARKS

Claims 1-44 and 51-61 are pending. Claims 2-5, 10-20, 23-27, 29-44, and 51-60 are withdrawn from consideration. Claims 1-5, 10-14, 23-27, 31-35, and 37-61 have been canceled. Claims 6-9, 15-22, 28 and 29 have been amended. Claims 6-9, 15-22, 28-30 and 36 remain in the case.

A request for reconsideration and review is pending, vis-à-vis a decision of December 12, 2002, on applicants' petition for withdrawal or modification of the restriction requirement. Pursuant to the mandate for a complete response, applicants present the following remarks, relating to claim revisions proffered above, but they do not waive their position set out in the request for reconsideration, for which a favorable disposition again is requested.

Responsive to the restriction requirement in question, applicants have canceled 1-5, 10-16, 19, 20, 23-27, 31-35 and 37-61. The basis for restriction between Groups V and X was that the nucleic acid claims read on "DNA encoding a (any) derivative that induces apoptosis and Oltvai *et al.* teach DNA molecule encoding a protein (derivative) that induce apoptosis." The claims have been amended to recite nucleic acid molecules that correspond substantially to SEQ ID NO:10 or that have at least about 45% or greater similarity to SEQ ID NO:10. Oltvai *et al.* disclose a protein that has less then 15% identity to the SEQ ID NO:10. Accordingly, the claims as amended recite a technical feature that defines a contribution over the molecule disclosed in Oltvai *et al.* The basis for restricting between the nucleic acid and protein claims has been obviated, and therefore examination of Groups V and X together is warranted. Reconsideration and withdrawal of the restriction between Groups V and X is respectfully requested.

Claims 6-9, 21, 22, 28 and 61 are objected to because they have not been amended to reflect the elected invention. The claims have been so amended. Claim 61 is objected to as depending on a non-elected claim and has been canceled.

The specification has been amended to remove the embedded hyperlink on page 20, and to remove the reference to Table 1 on page 63.

Claims 6-9, 21, 22, 28 and 61 are rejected under the second paragraph of Section 112, as being indefinite. The claims have been amended to address the concerns raised by the examiner. Reconsideration and withdrawal of the rejection under the second paragraph of Section 112 is respectfully requested.

Claims 1, 6-9, 21, 22 and 28 are rejected under the first paragraph of Section 112, on "written description" grounds. Thus, the examiner contains that the rejected claims contain subject matter that the specification does not describe in such a way as to reasonably convey to one skilled in the art that, at the time the invention was filed, the inventors possessed the claimed invention.

In this regard, the examiner notes that both human and murine Bim genes encode three different products, and he alleges that these products differ in both molecular structure and biological function. To substantiate the contention of differing biological function, the examiner invokes data in applicants' specification to urge that the degree of apoptosis varies among the various species. This in no way contravenes applicants' teaching and corresponding claim recitation, however, which is characterized by the ability to induce apoptosis.

The examiner also asserts that data in the specification, said to show that apoptosis is induced, relate to Bims and Bim$_L$ rather than to Bim$_{EL}$, the elected species. Attached to this response, however, are data demonstrating the binding properties in Table A and killing activity of Bim$_{EL}$. The data clearly show that Bim$_{EL}$, as taught in the specification, induces apoptosis, which is referenced as "killing activity" in Table B. The data further show that human Bim$_{EL}$ loses its apoptosis-inducing activity when the BH3 domain is deleted. In other words, a significant level of apoptosis-inducing activity is localized to the BH3 region. Furthermore, a variant of Bim$_{EL}$ has been generated that has lost the ability to bind to the dynein light chain. This variant exhibits improved apoptotic activity.*

* If requested, applicants will provide a declaration under Rule 132 which includes the referenced data.

The assays used to test the functional activity of these molecules are described briefly in the figure legend. More detailed descriptions of the assays appear in Puthalakath *et al.*, *Molecular Cell* 3: 287 (1999), and O'Connor *et al.*, *EMBO J.* 17: 384 (1998), copies of which accompany this response. These are standard assays for apoptosis induction and were well known before the priority date of the present application. The appended data provide conclusive evidence that (i) the human BimEL, according to SEQ ID NO:10, induces apoptosis, and (ii) variants of BimEL, generated in accordance with the present specification, function even better with respect to inducing apoptosis than the parent molecule.

Claims 21, 22 and 28 also stand rejected for alleged lack of enablement, with the examiner contending that data cannot be extrapolated, given the fact that even slight variations in protein's structure can have a significant and unpredictable effect on biological activity. The manufacture of other variants consistent with applicants' teachings, however, and their testing using the well known assays referenced above is well within the ambit of routine skill in this art and, hence, does not require experimentation that is "undue," within the meaning of the "enablement" provision of Section 112. More particularly, a skilled technician would be able to make nucleic acids that encode polypeptides based on SEQ ID NO:10 in which at least one amino acid is added, substituted or deleted, and then test the results using the available assays. This is borne out by the appended data.

The examiner comments on the correlation between binding to the dynein light chain and functionality. In particular, she notes that "the specification says that BH3 domain is essential for apoptosis," citing Example 7. The relevance of this relationship between the BH3 domain and the dynein light chain binding region of Bim are clearly disclosed in the specification, and it is submitted that the examiner may have misapprehended the import of Example 7. Although this example shows that BH3 plays a significant role in promoting apoptosis, at page 64, lines 29-30, it clearly is stated that BimL in which the BH3 domain is deleted is *not completely inactive*. The top of page 65 goes on to state that "thus, regions of Bim other than BH3 may promote apoptosis or interfere with clonogenicity in another

way." It is for this reason that the mutant which exhibits the deletion of the BH3 domain still retains some apoptotic activity.

The comments in the Action suggest that the examiner may not fully appreciate the functional significance of the BH domain and the dynein light chain binding domain in the context of various Bim isoforms. The BH domain, and more particularly the BH3 domain, are important with respect to Bcl-2 binding and therefore impact apoptosis by blocking the functioning of the pro-survival molecule Bcl-2. The dynein light chain domain binds to Bim. This prevents Bim from binding to Bcl-2 and preventing the pro-survival functioning of Bcl-2. In other words, the specification teaches that modulation of the dynein light chain region so that the Bim molecule can not longer bind can be used to design of variants optimized for apoptosis functionality.

The examiner further asserts that the specification does not teach any other structures responsible for apoptosis nor provide guidance as to what changes can be made to retain apoptotic activity while abolishing the dynein light chain ability of human Bim$_{EL}$. However, the specification does provide an explicit teaching in this regard at page 36 lines 20-22, which states that the region of the human Bim$_{EL}$ amino acid sequence which binds the dynein light chain is defined by residue numbers 42-131. Accordingly, the specification clearly teaches which mutations would be required in order to abolish dynein light chain binding. The specification further teaches at page 36, lines 4-10, that a Bim molecule which is unable to bind with dynein light chain is thereby free to interact with Bcl-2 and prevent its pro-survival function. Accordingly, this clearly teaches that a variant of Bim$_{EL}$, mutated in the amino acid residue region 42-131 such that it does not bind the dynein light chain, will inherently exhibit improved apoptotic inducing activity due to its ability to interact with and thereby inhibit the pro-survival molecule Bcl-2. This is confirmed by the appended data.

Finally, the examiner comments on disclosure in the specification that the biological functions of one splice variant of Bim are not the same as those of other splice variants, with longer splice variants having all of the amino acid sequence of shorter splice variants, but with the shorter splice variants having the best apoptotic activity. The reason that the

shorter splice variant has the best apoptotic activity is that this variant does not exhibit a dynein light chain binding region. This is disclosed in the specification, and further supports the notion that in designing variants to fall with the scope of the claims one would direct the variations to the disclosed dynein light chain binding region and seek to abolish the activity of this region to bind with this molecule, thereby freeing the Bim variant to bind to and inhibit Bcl-2.

Claims 1, 6-9, and 61 are rejected under the first paragraph of Section 112. The examiner states that the specification enables the production and use of SEQ ID NO:9 DNA that encodes SEQ ID NO:10, a human Bim_{EL} that is able to induce apoptosis, but does not enable "any other isolated DNA molecules." As noted above, the specification clearly provides guidance in this regard, enabling a skilled technician to make other DNAs and to implement assays to test the pro-apoptotic functional activity of these molecules. Moreover, the specification discloses results with other structurally similar molecules, human Bim_L, which differs from human Bim_{EL} slightly in length, and murine Bims, Bim_L, and Bim_{EL}, which also are highly similar to Bim_{EL}. Murine and human Bim_{EL} molecules exhibit 87% identity at the amino level, which is significantly higher than the closest prior art molecules identified via a Blast search, which exhibited less than 15% amino acid identity. Accordingly, the specification discloses a family of five Bim molecules that exhibit significant structural similarity at the protein level and, inherently, significant similarity at (i) the level of nucleic acid molecules which encode these proteins and (ii) the functional level.

The examiner alleges that the specification teaches Bim genes encoding different products with different molecular structures and different biological functions. To the contrary, the specification discloses a family of molecules that exhibit the same primary biological function, the ability to induce apoptosis. Further, the only significant structural difference between these molecules is the absence, in the case of Bims molecule, of a dynein light chain binding region. The absence of this region facilitates interaction of Bims with Bcl-2, thereby promoting a high level of apoptotic activity due to inhibition of Bcl-2 functioning. The structurally and functionally related family of Bim molecules taught by

applicants provides significant information in relation to the structure of these molecules in terms of functional impact. The specification also provides sufficient teaching to enable one to routinely design and perform procedures which would enable the expression of a nucleic acid sequence and testing of the pro-apoptotic functional activity of the protein products produced thereby.

On page 12 of the Action, the examiner states that the specification does not teach any method of using any peptide encoded by SEQ ID NO:9 that does not possess apoptotic activity. This statement is not understood, as applicants' claims do not encompass peptides that do not exhibit apoptotic activity.

Claims 1, 6-9, 21, 22, 28 and 61 are rejected under Section 101. The claims have been amended to recite "isolated" nucleic acids and polypeptides, obviating this rejection.

Claims 1 and 61 are rejected under Section 102(b) based on Oltvai *et al.* Oltvai *et al* does not disclose sequences as recited in the amended claims. Reconsideration and withdrawal of this rejection is requested.

In view of the foregoing amendments and remarks, it is believed that all claims are in condition for allowance. Reconsideration of all rejections and a notice of allowance are respectfully requested. Should there be any questions regarding this application, the examiner is invited to contact the undersigned attorney at the phone number listed below.

Respectfully submitted,

February 24, 2003
Date

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MARKED-UP VERSION SHOWING CHANGES MADE – SPECIFICATION

Please amend the last paragraph on page 20 as follows:

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particular preferred embodiment, nucleotide and sequence comparisons are made at the level of identity rather than similarity. Any number of programs are available to compare nucleotide and amino acid sequences. Preferred programs have regard to an appropriate alignment. One such program is Gap which considers all possible alignment and gap positions and creates an alignment with the largest number of matched bases and the fewest gaps. Gap uses the alignment method of Needleman and Wunsch. Gap reads a scoring matrix that contains values for every possible GCG symbol match. GAP is available on the ANGIS (Australian National Genomic Information Service) [at] website [http://mell.angis.org.au].

Please amend the first paragraph on page 63 as follows:

We next explored whether all isoforms of Bim were equivalent. An FDC-P1 clone expressing human Bcl-2 was transfected with vectors expressing Bim_{EL}, Bim_L or Bim_S and puromycin-resistant clones that expressed the same amount of each isoform were selected for further analysis (Figure 6A). To test for association with Bcl-2, immunoprecipitates prepared from cell lysates using a monoclonal antibody specific for human Bcl-2 were fractionated electrophoretically and blotted with anti-EE antibody. Each of the Bim isoforms clearly bound to Bcl-2 (Figure 6B). However, when the transfectants were deprived of IL-3 or subjected to γ-irradiation, it became evident that Bim_S antagonised Bcl-2 more effectively than Bim_L while Bim_{EL} was the least potent (Figures 6C). In addition, Bim_S suppressed L929 colony formation more effectively than Bim_L or Bim_{EL} [(Table 1)].

Thus, although all three Bim isoforms can bind to Bcl-2, they vary in cytotoxicity, Bims being the most potent.

MARKED-UP VERSION SHOWING CHANGES MADE – CLAIMS

Please cancel claims 1-5, 10-14, 23-27, 31-35, and 37-61 and amend the remaining claims as follows:

6. (Amended) [A] An isolated nucleic acid molecule [according to claim 1 wherein said nucleic acid molecule comprises] comprising a nucleotide sequence encoding or complementary to a sequence encoding an amino acid sequence [substantially as set forth in one] of SEQ ID NO:[8 or] 10 [or a derivative or homologue thereof] or having at least about 45% or greater [similarity] identity to [one or more of] SEQ ID NO:[8 or] 10 [or derivative or homologue thereof] wherein said amino acid sequence is characterized by the ability to induce apoptosis.

7. (Amended) [A] An isolated nucleic acid molecule [according to claim 1] comprising a nucleotide sequence [substantially as set forth in] of SEQ ID NO:[7 or] 9 [or a derivative or homologue thereof] or capable of hybridising to [one of] SEQ ID NO:[7 or] 9 under [low] moderate stringency conditions [at 42°C] wherein said nucleic acid molecule encodes a polypeptide characterized by the ability to induce apoptosis.

8. (Amended) [A] An isolated nucleic acid molecule according to claim 7 which further encodes an amino acid sequence corresponding to an amino acid sequence [substantially as set forth in one] of SEQ ID NO:[8 or] 10 [or a derivative or homologue thereof] or having at least about 45% or greater [similarity] identity to [one or more of] SEQ ID NO:[8 or] 10 [or a derivative of homologue thereof].

9. (Amended) [A] An isolated nucleic acid molecule according to claim 7 [substantially as set forth in one] of SEQ ID NO:[7 or] 9.

15. (Amended) [A] An isolated polypeptide [according to claim 10] comprising an amino acid sequence [substantially as set forth in] of SEQ ID NO:[8 or] 10 [or derivative or homologue thereof] or a sequence having at least about 45% [similarity] identity to [one or more of] SEQ ID NO:[8 or] 10, wherein said polypeptide is characterized by the ability to induce apoptosis.

16. (Amended) [A] An isolated polypeptide according to claim [10] 15 encoded by a nucleotide sequence [substantially as set forth in] of SEQ ID NO:[7 or] 9 [or a derivative or homologue thereof] under [low] moderate stringency conditions [at 42°C].

17. (Amended) [A] An isolated polypeptide according to claim 16 further comprising an amino acid sequence [substantially as set forth in] of SEQ ID NO:[8 or] 10 [or derivative or homologue thereof] or a sequence having at least about 45% [similarity] identity to [one or more of] SEQ ID NO:[8 or] 10.

18. (Twice Amended) [A] An isolated polypeptide according to claim 16 [substantially as set forth in] having SEQ ID NO:[8 or] 10.

19. (Twice Amended) [A] An isolated polypeptide according to claim [10] 15 in homodimeric form.

20. (Twice Amended) [A] An isolated polypeptide according to claim [10] 15 in heterodimeric form.

21. (Twice Amended) A variant of an isolated [*Bim*] nucleic acid molecule as claimed in claim [1] 6 comprising one or more nucleotide mutations in said nucleic acid molecule resulting in at least one amino acid addition, substitution and/or deletion to the polypeptide encoded by said variant wherein said polypeptide cannot bind, couple or otherwise associate with a dynein light chain and wherein said polypeptide is characterized by the ability to induce apoptosis.

22. (Amended) A variant according to claim 21 wherein said mutation results in an amino acid addition, substitution and/or deletion in the region of the polypeptide chain which binds the dynein light chain.

28. (Amended) A variant according to claim 22 wherein said [*Bim*] nucleic acid molecule is human *Bim*$_{EL}$ and said region is defined by amino acid residue numbers 42 to 131.

29. (Amended) A variant of an isolated [Bim] polypeptide as claimed in claim [10] 15 comprising at least one amino acid addition, substitution, and/or deletion wherein said variant cannot bind, couple or otherwise associate with the dynein light chain.

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Came w/ Paper # 19

Bim: a nov l member of the Bcl-2 family that promotes apoptosis

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Certain members of the Bcl-2 family inhibit apoptosis while others facilitate this physiological process of cell death. An expression screen for proteins that bind to Bcl-2 yielded a small novel protein, denoted Bim, whose only similarity to any known protein is the short (nine amino acid) BH3 motif shared by most Bcl-2 homologues. Bim provokes apoptosis, and the BH3 region is required for Bcl-2 binding and for most of its cytotoxicity. Like Bcl-2, Bim possesses a hydrophobic C-terminus and localizes to intracytoplasmic membranes. Three Bim isoforms, probably generated by alternative splicing, all induce apoptosis, the shortest being the most potent. Wild-type Bcl-2 associates with Bim *in vivo* and modulates its death function, whereas Bcl-2 mutants that lack survival function do neither. Significantly, Bcl-x_L and Bcl-w, the two closest homologues of Bcl-2, also bind to Bim and inhibit its activity, but more distant viral homologues, adenovirus E1B19K and Epstein–Barr virus BHRF-1, can do neither. Hence, Bim appears to act as a 'death ligand' which can only neutralize certain members of the pro-survival Bcl-2 sub-family.

Keywords: apoptosis/Bcl-2/BH3/Bim/protein–protein interaction

Introduction

Apoptosis, the physiological process of cell death, is critical for modelling tissues and maintaining homeostasis in multicellular organisms (Kerr *et al.*, 1972; Jacobson *et al.*, 1997). The mechanism of this intrinsic suicide programme is under intense scrutiny. The executioners are a set of cysteine proteinases, termed caspases, that degrade critical cellular substrates (Nicholson and Thornberry, 1997). The regulatory machinery that governs the activation of the caspases is less well understood, but a central role is played by the Bcl-2 family (Cory, 1995; Korsmeyer, 1995; White, 1996; Jacobson, 1997; Kroemer, 1997; Reed, 1997). Bcl-2 itself was the first intracellular regulator of apoptosis to be identified (Vaux *et al.*, 1988), and high levels enhance cell survival under diverse cytotoxic conditions (Sentman *et al.*, 1991; Strasser *et al.*, 1991). Other cellular homol gues, such as Bcl-x_L (Boise *et al.*, 1993) and Bcl-w (Gibson *et al.*, 1996), also enhance cell survival, as do more distantly related viral homologues, such as the adenovirus E1B19K protein (White *et al.*, 1992) and Epstein–Barr virus (EBV) BHRF-1 (Henderson *et al.*, 1993). Remarkably, the family also includes members such as Bax (Oltvai *et al.*, 1993) and Bak (Chittenden *et al.*, 1995b; Farrow *et al.*, 1995; Kiefer *et al.*, 1995) which antagonize the activity of the pro-survival proteins and provoke apoptosis when expressed at high concentrations.

The ability of the pro-survival and anti-survival family members to form heterodimers makes it possible that each type might titrate the other, potentially accounting for their opposing actions. The relative concentrations of the opposing sub-family members would then determine whether the cell lives or dies (Oltvai *et al.*, 1993; Oltvai and Korsmeyer, 1994). Mutagenesis of Bcl-2 initially suggested that its ability to inhibit cell death required binding to a pro-apoptotic family member (Yin *et al.*, 1994), but Bcl-x_L mutants have been identified that do not bind Bax or Bak yet still block apoptosis (Cheng *et al.*, 1996). Thus, it remains unclear whether the ability to associate with other family members is central to regulating apoptosis.

The homology between members of the Bcl-2 family is greatest within four small segments, designated Bcl-2 homology (BH) regions (Yin *et al.*, 1994; Chittenden *et al.*, 1995a; Gibson *et al.*, 1996; Zha *et al.*, 1996), some of which contribute to the interactions between Bcl-2 family members. The N-terminal BH4 domain is restricted to some antagonists of apoptosis, while BH1, BH2 and BH3 can be found in both sub-families. Association of a pro-survival with a pro-apoptotic protein, such as Bcl-2 (or Bcl-x_L) with Bax (or Bak), requires the BH1 and BH2 domains of the former (Yin *et al.*, 1994; Hanada *et al.*, 1995; Sedlak *et al.*, 1995) and the BH3 domain of the latter (Chittenden *et al.*, 1995a; Simonian *et al.*, 1996; Zha *et al.*, 1996). In the tertiary structure of Bcl-x_L, the BH1, BH2 and BH3 domains form an elongated hydrophobic cleft (Muchmore *et al.*, 1996) along which the amphipathic helix formed by BH3 domains of the pro-apoptotic proteins can bind (Sattler *et al.*, 1997). The importance of the BH3 region for facilitating apoptosis has been underscored by the discovery of several BH3-containing proteins: Bik/Nbk (Boyd *et al.*, 1995; Han *et al.*, 1996), Bid (Wang *et al.*, 1996) and Hrk/DP5 (Imaizumi *et al.*, 1997; Inohara *et al.*, 1997), which are otherwise unrelated to the Bcl-2 family but are potent activators of apoptosis when overexpressed.

To search for additional regulators of apoptosis, we have screened a cDNA expression library with a Bcl-2 protein probe. This interaction screen has yielded a novel BH3-containing protein, which we have denoted Bim. Three Bim isoforms all pr mote apoptosis but differ in potency. Bim interacts with some but not all Bcl-2 family

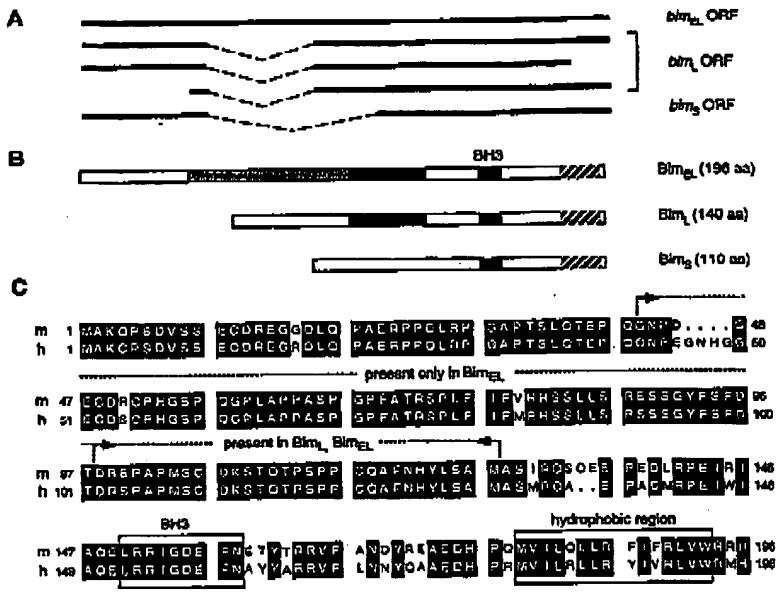

Fig. 1. Isolation of cDNAs encoding three isoforms of Bim: Bim_{EL}, Bim_L and Bim_S. (A) Open reading frames of five independent clones isolated by screening a cDNA expression library with recombinant Bcl-2 protein. Dotted lines indicate putative splices. (B) Relationship of the three Bim isoforms. The black box denotes the BH3 homology region and the hatched box the predicted hydrophobic region. Regions specific to the larger splice variants are shaded. (C) Sequence alignment of the mouse and human Bim_{EL} polypeptide sequences using the GCG 'BESTFIT' program, identical residues appear on a dark background. The BH3 homology region and the C-terminal hydrophobic region predicted by the Kyte–Doolittle algorithm are boxed. The arrows indicate residues present only in the longer isoforms. Since the nucleotide sequences of the mouse and human cDNAs diverged 5′ of the predicted initiating ATG and there are stop codons in all three reading frames upstream of the human open reading frame, that start codon is likely to be correct.

members that promote cell survival, and only those pro-survival relatives that bind to it can neutralize its cytotoxicity. Bim therefore appears to represent a death ligand with a specificity restricted to certain pro-survival members of the Bcl-2 family.

Results

Isolation of a novel gene encoding a Bcl-2-binding protein

In an attempt to identify novel proteins that bind to Bcl-2, we used recombinant human Bcl-2 protein, labelled with ^{32}P (Blanar and Rutter, 1992), to screen a bacteriophage λ cDNA expression library constructed from the $p53^{-/-}$ T lymphoma cell line KO52DA20 (Strasser *et al.*, 1994). A screen of 10^6 clones yielded five independent clones that encoded the same novel protein, which we named Bim, for Bcl-2 interacting mediator of cell death. Sequence analysis of the *bim* cDNAs revealed three variants of the coding region, apparently produced by alternative splicing, that we designated bim_{EL}, bim_L and bim_S (Figure 1A). RT–PCR on mRNA from KO52DA20 cells gave PCR products of the sizes expected for each of these transcripts, although bim_S was in low yield (data not shown). The predicted Bim_{EL}, Bim_L and Bim_S proteins comprise 196, 140 and 110 amino acid residues (Figure 1B). Hybridizing human fetal spleen and peripheral blood cDNA libraries with a mouse *bim* cDNA yielded human cDNAs encoding Bim_L and Bim_{EL}. Human Bim_{EL} is a protein of 198 residues, 89% identical to its mouse counterpart (Figure 1C), and human Bim_L (138 residues) is 85% identical to mouse Bim_L.

Bim has no substantial homology with any protein in current databases. However, scrutiny of its sequence (Figure 1C) revealed a stretch of nine amino acids corresponding to a BH3 homology region (Boyd *et al.*, 1995; Chittenden *et al.*, 1995a). The BH3 region of Bcl-x_L and a peptide corresponding to this region of Bak have each been shown to form part of an amphipathic α helix (Sattler *et al.*, 1997). Plotting the BH3 region of Bim as a helical wheel revealed that it was also amphipathic (data not shown). Apart from the BH3 region, the Bim sequence is unrelated to that of any other BH3-containing protein; it contains no other BH region, nor indeed any other known functional motif. The protein does, however, have a C-terminal hydrophobic region (Figure 1C). Such regions are found in most members of the Bcl-2 family and appear to be important for their localization to intracytoplasmic membranes (Kroemer, 1997).

Northern blot analysis showed that *bim* was expressed in a number of B- and T-lymphoid cell lines, although not in the myeloid line FDC-P1(Figure 2). A major transcript of 5.7 kb and minor transcripts of 3.8, 3.0 and 1.4 kb were detected. Neither the level nor relative abundance of these transcripts changed significantly in KO52DA20 cells induced to undergo apoptosis by treatment with dexamethasone (Figure 2, compare lanes 1 and 2, and lanes 3 and 4) or exposure to γ-radiation (compare lanes 3 and 5). In addition, overexpression of *bcl-2* in several of the lines did not affect *bim* mRNA levels (Figure 2).

Bim localiz s to cytoplasmic membranes

The presence of the C-terminal hydrophobic domain in Bim prompted us t investigate its subcellular localization.

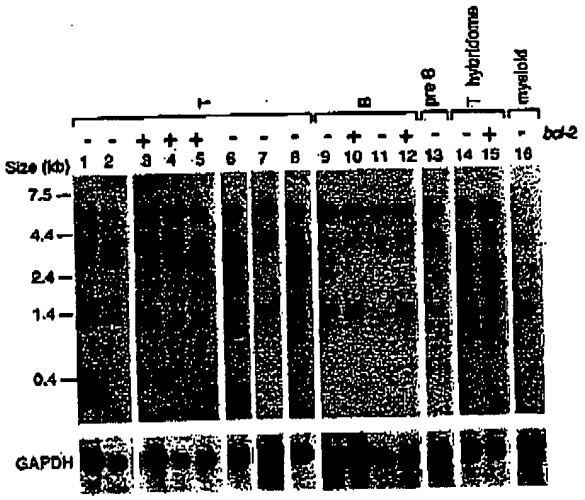

Fig. 2. Expression of *bim* RNA in haemopoietic cell lines. Northern blot analysis of poly(A)$^+$ RNA, using a mouse *bim* cDNA probe. The RNAs were derived from the following mouse lines: T-lymphomas KO52DA20 (lanes 1–5), WEHI 703 (lane 6), WEHI 707 (lane 7) and WEHI 7.1 (lane 8), B-lymphomas CH1 (lanes 9 and 10) and WEHI 231 (lanes 11 and 12), pre-B-lymphoma WEHI 415 (lane 13), T-hybridoma B6.2.16 BW2 (lanes 14 and 15) and myeloid progenitor FDC-P1 (lane 16). Those lines that harbour a *bcl*-2 expression vector or transgene are indicated by a plus sign. Certain RNAs were isolated from cells exposed to cytotoxic conditions: 1 μM dexamethasone (lanes 2 and 4), γ-irradiation (10 Gy) (lane 5). Lanes from a single autoradiograph have been electronically arranged in order.

L929 fibroblasts were transiently transfected with an expression vector encoding Bim_L tagged with an N-terminal EE epitope, and the permeabilized cells were stained with an anti-EE monoclonal antibody. Confocal microscopy revealed that Bim_L had a granular cytoplasmic distribution, consistent with an association with intracellular membranes (Figure 3A). When we also introduced the bim_L vector into L929 cells stably expressing human Bcl-2, the similarity of the anti-EE staining pattern of these cells (Figure 3C) to that of those expressing Bim_L alone (Figure 3A) demonstrated that high concentrations of Bcl-2 did not perturb the localization of Bim_L. The pattern of Bim_L staining was similar to that reported for Bcl-2 (Monaghan *et al.*, 1992; Krajewski *et al.*, 1993; Lithgow *et al.*, 1994), and overlaying the images obtained from the same cells stained with anti-Bcl-2 (Figure 3B) and anti-EE (Figure 3C) antibodies showed that the two proteins co-localized (Figure 3D). This co-localization does not simply reflect binding of Bim to Bcl-2 since a mutant form of Bim lacking the BH3 region, and therefore incapable of binding to Bcl-2 (see below), localized similarly (Figure 3E).

Subcellular fractionation studies in FDC-P1 cells (see below) were consistent with these observations. Immunoblotting of fractionated lysates obtained from cell lines overexpressing human Bcl-2 and either EE-tagged Bim_L or EE-tagged Bim_L lacking the BH3 region showed that all three proteins were present in the nuclear and membrane fractions but not the cytosolic fraction (Figure 3F and data not sh wn). Together with the microscopic data, these results suggest that Bim localizes to intracytoplasmic membranes, independently of its association with Bcl-2.

Overexpression of Bim kills cells by a pathway requiring caspases

Other known 'BH3-only' proteins (Bik/Nbk, Bid and Hrk/DP5) provoke apoptosis when highly expressed (Boyd *et al.*, 1995; Han *et al.*, 1996; Wang *et al.*, 1996; Imaizumi *et al.*, 1997; Inohara *et al.*, 1997). We therefore tested whether Bim is cytotoxic by transiently transfecting 293T human embryonal kidney cells with a plasmid encoding EE-Bim_L. The viability of the transfected cells was determined by flow cytometric analysis of permeabilized cells stained with anti-EE antibody and the DNA-intercalating dye propidium iodide (PI). Whereas almost all untransfected cells or those transfected with an empty vector remained viable after 24 h, many of those expressing Bim (i.e. EE antibody-positive) contained sub-diploid DNA (Figure 4A). Indeed, by 3 days, 90% of the cells expressing Bim_L were dead (Figure 4B). The extent of cell death increased with the amount of *bim* DNA transfected (black bars, Figure 4C).

The cells expressing Bim appeared to die by apoptosis, as assessed by cell morphology and the generation of sub-diploid DNA (Figure 4A). As expected, the death process required activation of caspases, because co-expression of baculovirus p35, a competitive inhibitor of many types of caspases (Bump *et al.*, 1995), antagonized Bim-induced cell death, whereas an inactive (D87E) mutant p35 (Xue and Horvitz, 1995) did not (Figure 4C). Since CrmA, a potent inhibitor of caspases 1 and 8 (ICE and FLICE) (Orth *et al.*, 1996; Srinivasula *et al.*, 1996), was not effective (Figure 4C), these particular caspases do not appear to play an essential role.

Numerous failed attempts to generate lines that stably express Bim suggested that it is toxic to diverse cell types. Those repeatedly tested include haemopoietic lines (FDC-P1, CH1, Jurkat, SKW6 and B6.2.16BW2), fibroblastoid lines (Rat-1, NIH-3T3 and L929) and an epithelial line (293). The cells were electroporated with a vector encoding antibiotic resistance and either EE- or FLAG-tagged Bim_L, but no drug-selected line that expressed Bim emerged. A vector encoding untagged Bim also failed to generate viable clones. We quantified the cytotoxicity of Bim by colony assays on transfected L929 fibroblasts. Cells transfected with the EE-Bim_L vector yielded only one-fifth as many antibiotic-resistant colonies as those transfected with the control vector, and when six of the EE-Bim_L-transfected, drug-resistant colonies were expanded, only one contained any Bim and the level was very low (Table I and data not shown). Thus, Bim suppresses clonogenicity, and expression above a relatively low threshold is incompatible with prolonged cell viability.

Bim cytotoxicity can be abrogated by wild-type Bcl-2 but not inactive mutants

Co-expression experiments established that Bcl-2 could block cell death induced by Bim_L (Figure 4D). In 293T cells transiently transfected with both the *bcl*-2 and bim_L plasmids, relatively few cells died, even with a high concentration of bim_L DNA (compare the fourth bar in Figure 4C with the third in Figure 4D). The cytotoxicity of Bim, however, could not be countered by mutant forms of Bcl-2 rendered inactive by deletion of the BH4 homology region (ΔBH4) (Borner *et al.*, 1994; D.C.S.Huang, J.M.Adams and S.Cory, submitted), or by

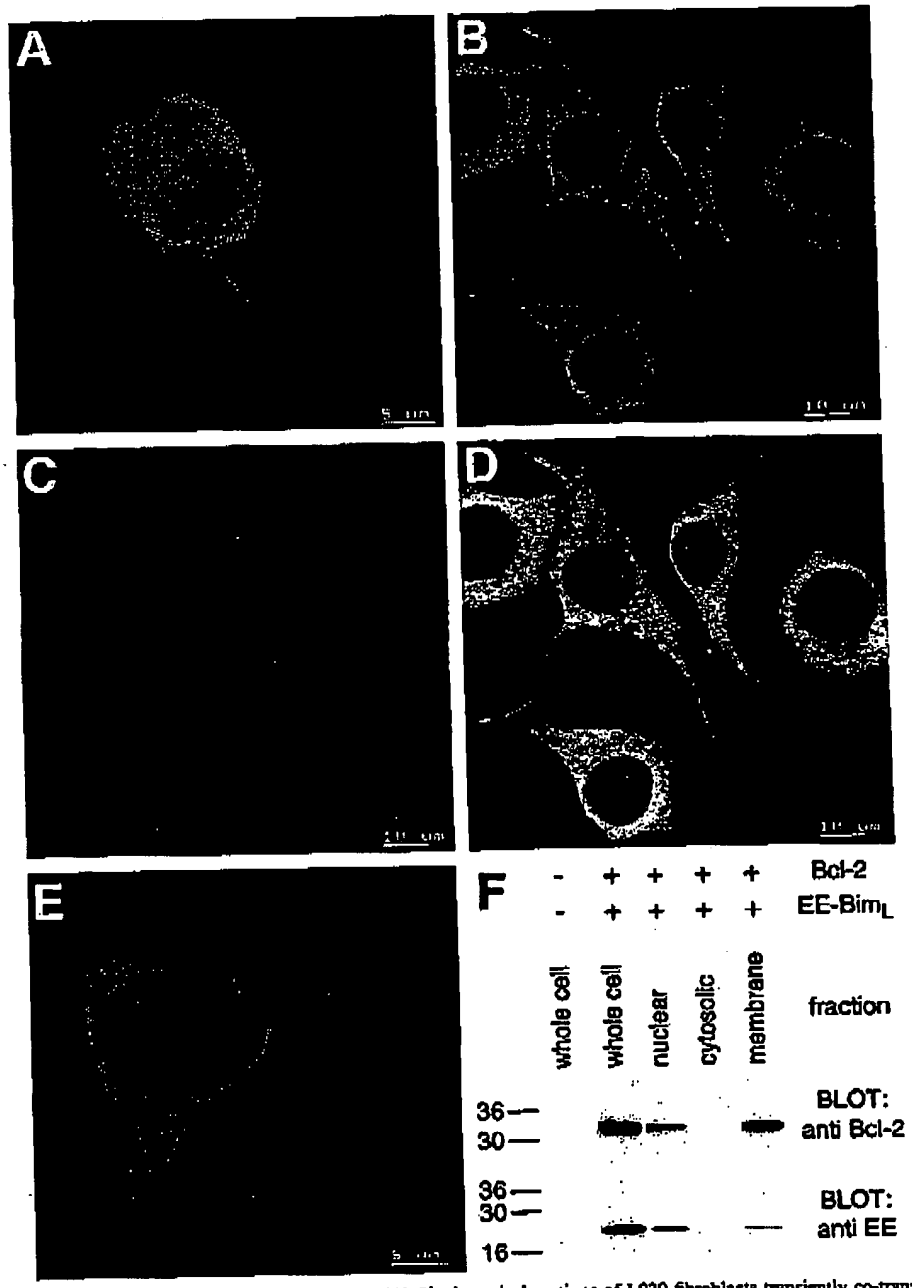

Fig. 3. Localization of Bim protein to intracellular membranes. (A) Single optical sections of L929 fibroblasts transiently co-transfected with EE-tagged Bim$_L$ and baculovirus p35, then stained with the anti-EE antibody. (B) and (C) Confocal images of L929 cells stably co-expressing human Bcl-2 and EE-tagged Bim$_L$ stained with anti-human Bcl-2 antibody (B) or anti-EE antibody (C). (D) Overlay of images (B) and (C). Co-localization of antibody staining is indicated by yellow fluorescence. (E) Confocal image of L929 cells transiently expressing human Bcl-2 and EE-tagged Bim ΔBH3 stained with anti-EE antibody. (F) Subcellular fractionation of lysates from FDC-P1 cells expressing EE-Bim$_L$ and Bcl-2. Lysates from equivalent numbers of unfractionated cells (whole) and of subcellular fractions (nuclear, cytoplasmic or membrane), were resolved by SDS–PAGE and immunoblotted using the anti-human Bcl-2 (upper panels) or anti-EE (lower panels) monoclonal antibodies.

a point mutation in its BH1 (G145E) or BH2 (W188A) region (Yin *et al.*, 1994) (Figure 4D). Thus the ability to antagonize Bim-induced cell death required a functional Bcl-2 molecule.

High levels of Bcl-2 allowed stable expression of Bim$_L$. Indeed, when retrovirally infected L929 clones overexpressing Bcl-2 were transfected with the EE-Bim$_L$ vector, the frequency of antibiotic-resistant colonies approached that obtained with the control vector, and four of six colonies analysed contained moderate to high levels of Bim (Table I and data not shown). Similarly, using FDC-P1 clones overexpressing wild-type Bcl-2 (but not

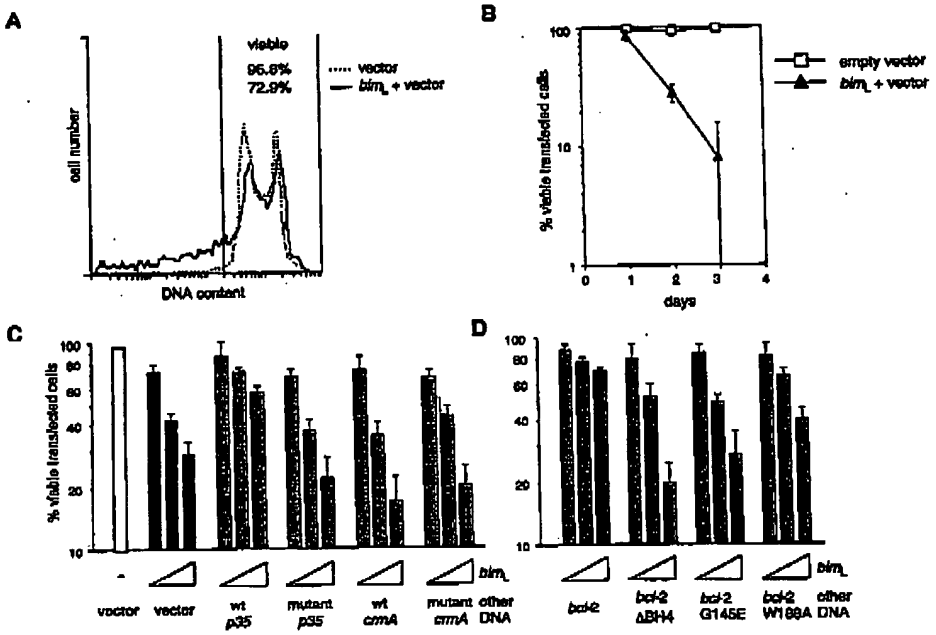

Fig. 4. Bim induces apoptosis which can be inhibited by the general caspase inhibitor p35 and Bcl-2 but not by CrmA. (A) Flow cytometric DNA analysis (see Materials and methods) of 293T cells transfected 24 h previously with EE-*bim*$_L$ plasmid (0.5 μg). (B) Kinetics of apoptosis elicited by EE-*bim*$_L$ plasmid (0.5 μg), assessed as in (A). (C) Cell viability 48 h after transfection with 0.1, 0.2 or 0.5 μg of EE-*bim*$_L$ plasmid alone (black bars) or together with 0.5 μg of wild-type or mutant *p35* or *crmA* plasmid (grey bars). (D) Cell viability 48 h after transfection with 0.1, 0.2 or 0.5 μg of EE-*bim*$_L$ plasmid together with 0.5 μg of the indicated wild-type or mutant *bcl-2* plasmids. (C) and (D) show the percentage of viable Bim-expressing cells, determined by DNA FACS analysis, as in (A), and are the means ± SD of three or more independent experiments.

Table I. Bim inhibition of L929 colony growth is abrogated by Bcl-2

Cell line	Construct	Cloning efficiency	No. of antibiotic-resistant clones expressing Bim
L929	control	1.0	0/6
	bim$_L$	0.21 ± 0.04	1/6
	bim$_{EL}$	0.19 ± 0.05	1/6
	bim$_S$	0.11 ± 0.03	0/6
	bim$_L$ ΔBH3	0.69 ± 0.07	6/6
L929 *bcl-2*	control	1.0	0/6
	bim$_L$	0.64 ± 0.07	4/6

Parental L929 fibroblasts and a cloned derivative that stably expresses human Bcl-2 (L929 *bcl-2*) were co-transfected with a plasmid conferring antibiotic resistance with or without encoding various forms of Bim. After 48 h, antibiotic selection was added and the number of colonies were scored after 14–18 days. The data shown are means ± SD of at least four independent experiments.

mutant Bcl-2), we could readily establish sub-clones expressing intermediate to high levels of Bim$_L$ (Figure 5A). When grown in the presence of interleukin-3 (IL-3), all were indistinguishable in growth characteristics and morphology from the parental FDC-P1 cells or those bearing Bcl-2 alone. However, when deprived of IL-3 or irradiated, cells expressing Bcl-2 and a moderate or high level of Bim died more rapidly than those expressing Bcl-2 alone (Figure 5B). Since each clone had the same level of Bcl-2 (not shown), their sensitivity to apoptosis presumably reflects the ratio of the pro-death protein Bim to the pro-survival protein Bcl-2.

The three isoforms of Bim all interact with Bcl-2 in vivo but vary in cytotoxicity

We next explored whether all isoforms of Bim were equivalent. An FDC-P1 clone expressing human Bcl-2 was transfected with vectors expressing Bim$_{EL}$, Bim$_L$ or Bim$_S$, and antibiotic-resistant clones that expressed the same amount of each isoform were selected for further analysis (Figure 6A). To test for association with Bcl-2, immunoprecipitates prepared from cell lysates using a monoclonal antibody specific for human Bcl-2 were fractionated electrophorctically and blotted with anti-EE antibody. Each of the Bim isoforms clearly bound to Bcl-2 (Figure 6B). However, when the transfectants were deprived of IL-3 or subjected to γ-irradiation, it became evident that Bim$_S$ antagonized Bcl-2 more effectively than Bim$_L$, while Bim$_{EL}$ was the least potent (Figures 6C). In addition, Bim$_S$ suppressed L929 colony formation more effectively than Bim$_L$ or Bim$_{EL}$ (Table I). Thus, although all three Bim isoforms can bind to Bcl-2, they vary in cytotoxicity, Bim$_S$ being the most potent.

Bim binds to and antagonizes Bcl-x$_L$ and Bcl-w but not viral Bcl-2 homologues

Bim also associates with certain other anti-apoptotic Bcl-2 family members. Immunoprecipitation of lysates from ^{35}S-labelled 293T cells transiently co-transfected with the relevant vectors revealed binding to Bcl-x$_L$, although not to a mutant (mt 7) that lacks pro-survival activity, nor to two mutants (mt 1 and mt 15) that retain significant anti-apoptotic activity but cannot bind to Bax (Cheng *et al.*, 1996) (Figure 7A). Binding to Bcl-x$_L$ and Bcl-w was

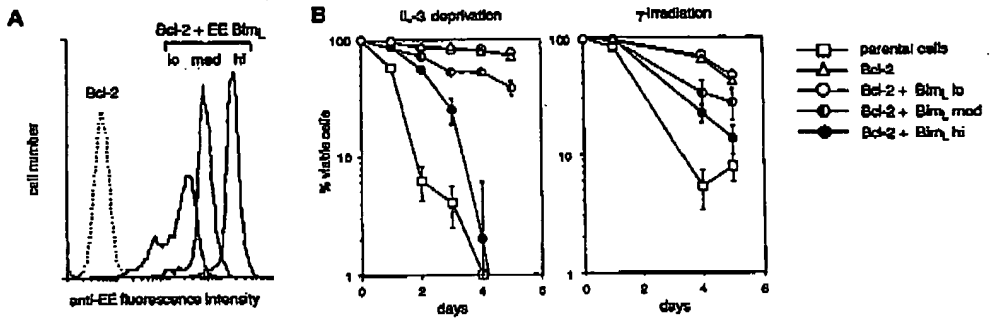

Fig. 5. Bim antagonizes the anti-apoptotic activity of Bcl-2 in a dose-dependent fashion. (A) Immunofluorescence staining of cloned FDC-P1 cell lines stably expressing Bcl-2 alone (dashed line) or co-expressing Bcl-2 and varying levels of EE-Bim$_L$ (solid lines). (B) Viability of these clones when cultured in the absence of IL-3 or after exposure to γ-irradiation (10 Gy). Cell viability was assessed by vital dye exclusion. Data shown are means ± SD of at least three experiments and are representative of results obtained with at least three independent lines of each genotype.

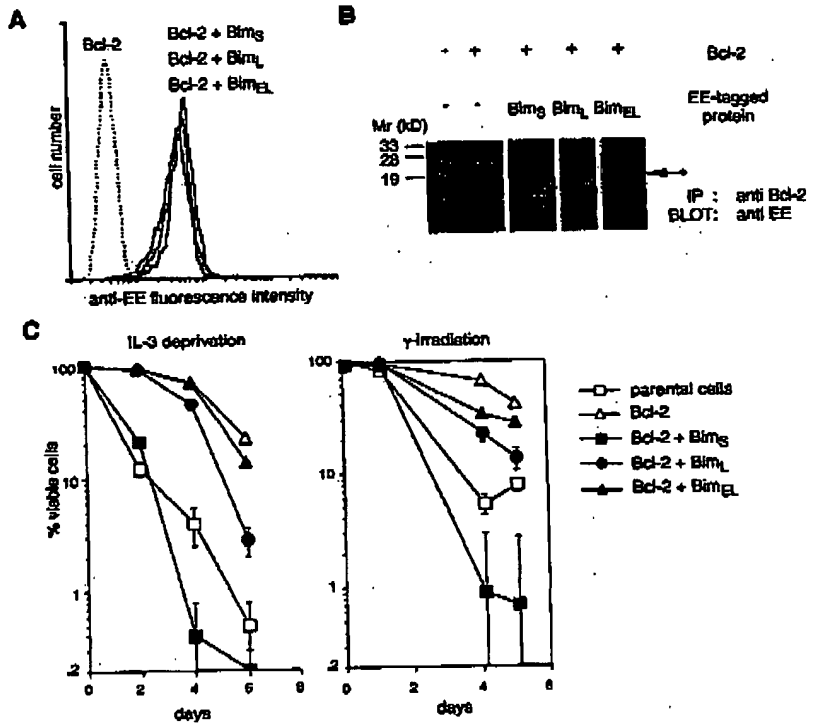

Fig. 6. Different killing activities of the three Bim isoforms. (A) Immunofluorescence staining, performed in parallel, of cloned FDC-P1 lines expressing Bcl-2 alone (dotted) or Bcl-2 plus EE-tagged Bim$_L$, Bim$_{EL}$ or Bim$_S$ (solid lines). (B) Association of EE-tagged Bim$_S$, Bim$_L$ and Bim$_{EL}$ with Bcl-2 demonstrated by anti-EE immunoblots of immunoprecipitates prepared with anti-human Bcl-2 monoclonal antibody from FDC-P1 cells expressing the indicated proteins. The 25 kDa non-specific band in the fourth lane, indicated by an asterisk, was not consistently seen. (C) Effect of Bim isoforms on viability of FDC-P1 cells expressing Bcl-2, after removal of growth factor or exposure to irradiation. All data were obtained on lines that expressed equivalent levels of the introduced proteins (see A). Those shown are means ± SD of at least three experiments representative of the results obtained with at least two independent lines of each genotype.

confirmed by immunoprecipitation followed by Western blot analysis (Figure 7B). Not all mediators of cell survival associate with Bim, however. Under the same conditions, Bim did not bind to either of two virally encoded Bcl-2 homologues, the adenovirus E1B19K protein and the EBV BHRF-1 protein (Figure 7B).

Functional tests mirrored the binding properties of the various Bcl-2 homologues. When transiently co-expressed with Bim in 293T cells, Bcl-x_L and Bcl-w countered Bim toxicity as effectively as Bcl-2 (compare Figures 7C and D with Figure 4D). In contrast, little inhibition was observed with comparable levels of the mutant Bcl-x_L proteins (Figure 7C) or the adenovirus E1B19K protein (Figure 7D). These data suggest that Bcl-2-like inhibitors of apoptosis must bind to Bim to inhibit its action.

Bim does not interact with any pro-apoptotic family member tested. No interaction of Bim with Bax could be observed under conditions in which Bim–Bcl-x_L association was readily detectable (Figure 7E). In other experiments (not shown) in which we co-expressed FLAG-

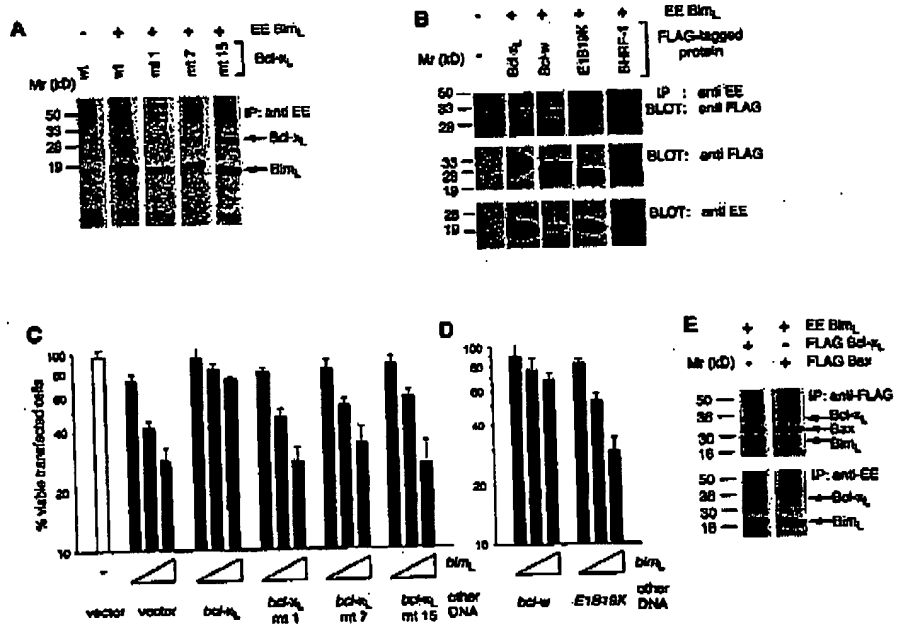

Fig. 7. Bim binds to and antagonizes Bcl-x_L or Bcl-w but not E1B19K. (A) Lysates of ^{35}S-labelled 293T cells transiently co-transfected with the plasmids encoding the indicated proteins were immunoprecipitated with anti-EE antibody, and the EE-Bim$_L$-containing complexes were fractionated by SDS–PAGE. (B) Lysates from parental 293T cells or 293T cells co-expressing EE-tagged Bim$_L$ and FLAG-tagged Bcl-x_L, Bcl-w, E1B19K or BHRF-1 were immunoblotted directly or after immunoprecipitation, as indicated. (C and D) 293T cells were transiently transfected with a vector control (unfilled bar) or with 0.1, 0.2 or 0.5 μg of EE-Bim$_L$ plasmid, either alone (black bars) or together with 0.5 μg of plasmids encoding wild-type or mutant Bcl-x_L (C), Bcl-w or E1B19K protein (D) (grey bars). The flow cytometric analysis was as described in the legend to Figure 4. Data shown are means ± SD of three or more independent experiments. (E) Lysates of ^{35}S-labelled 293T cells transiently co-transfected with plasmids encoding the indicated proteins were immunoprecipitated with either anti-FLAG or anti-EE antibody, and the resulting complexes were fractionated by SDS–PAGE.

tagged Bim with a range of other EE-tagged pro-apoptotic family members, we failed to detect any Bim homodimers or any interaction of Bim with Bak, Bad, Bik or Bid.

The BH3 region is essential for interaction of Bim with pro-survival Bcl-2 family members and for most of its ability to promote apoptosis

Since the BH3 region of several death-promoting proteins is essential for their activity (see Introduction), we tested a *bim*$_L$ mutant lacking the BH3 region. In transiently transfected 293T cells, the mutant protein (ΔBH3) was readily detected by immunoblotting but it did not bind to Bcl-x_L (Figure 8A), or to Bcl-2 or Bcl-w (data not shown).

In some biological assays, the ΔBH3 mutant of Bim appeared inert. In contrast to wild-type Bim, it was easy to establish lines expressing Bim$_L$ ΔBH3 from FDC-P1 (Figure 8B) or L929 cells (Table I and data not shown). Moreover, Bim$_L$ ΔBH3 did not impair the viability of the FDC-P1 cells in either the presence or absence of Bcl-2 (Figure 8C). Finally, 293T cells transiently transfected with Bim$_L$ ΔBH3 exhibited high viability (not shown). These results indicate that the BH3 region is critical for Bim to promote apoptosis and suggest that Bcl-2 blocks this activity of Bim by binding to that domain. Importantly, however, Bim$_L$ ΔBH3 was not completely inactive. In the L929 clonogenicity assay, it still markedly suppressed colony formation (Table I). Thus, regions of Bim other than BH3 may promote apoptosis or interfere with clonogenicity in another way, such as by blocking cell growth.

Discussion

Our screen for proteins that interact with Bcl-2 *in vitro* yielded the novel protein Bim. When overexpressed, Bim proved to be highly cytotoxic for diverse cell types. Indeed, Bim probably is a more potent inducer of cell death than any other known Bcl-2 family member, since stably overexpressing cell lines readily could be derived from the others (Boise *et al.*, 1993; Oltvai *et al.*, 1993; Boyd *et al.*, 1995; Chittenden *et al.*, 1995b; Farrow *et al.*, 1995; Kiefer *et al.*, 1995; Yang *et al.*, 1995; Han *et al.*, 1996; Wang *et al.*, 1996; Inohara *et al.*, 1997; and our unpublished observations). Bim-induced cytotoxicity could be diminished by co-expression of Bcl-2, Bcl-x_L or Bcl-w, all of which bound to Bim *in vivo*. On the other hand, no protection was provided by mutant forms of Bcl-2 and Bcl-x_L that did not bind to Bim, including two Bcl-x_L mutants that provide substantial protection against other apoptotic stimuli (Cheng *et al.*, 1996). Thus, the pro-apoptotic action of Bim must, at least in part, reflect its ability to complex with the anti-apoptotic members of the family.

The small BH3 domain is the only region of Bim exhibiting homology with other members of the Bcl-2 family (Figure 9A). As this region was essential for most of its cytotoxic action (see below), Bim can be considered together with Bik/Nbk, Bid and Hrk/DP5 as a group of 'BH3-only' pro-apoptotic proteins. As demonstrated for Bak, the BH3 region of each may form an amphipathic helix that interacts with the elongated hydrophobic cleft

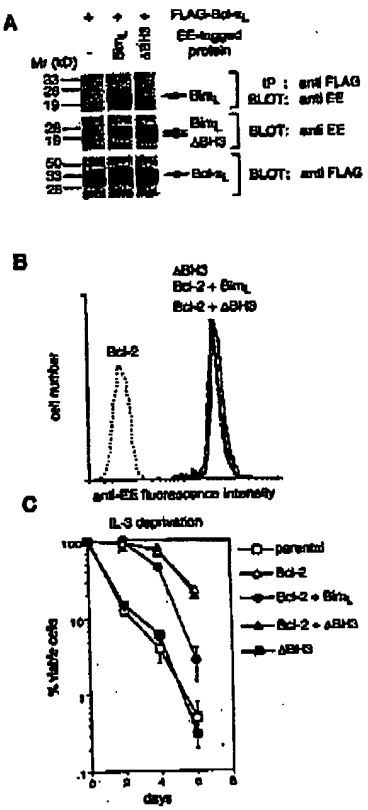

Fig. 8. The BH3 homology region of Bim is required for binding to and inhibiting pro-survival Bcl-2 homologues. (A) Immunoblot showing that Bcl-x_L associates with wild-type Bim_L but not with the ΔBH3 mutant. (B) Immunofluorescence staining of cloned FDC-P1 lines expressing Bcl-2 alone (dotted) or with EE-Bim_L or EE-Bim ΔBH3 (solid lines), and of EE-Bim ΔBH3 in the parental FDC-P1 cells (broken line). (C) Viability of FDC-P1 clones expressing the indicated proteins (see B) was assessed by vital dye exclusion. Data shown are means ± SD of at least three experiments and are representative of results obtained with at least three independent lines of each genotype.

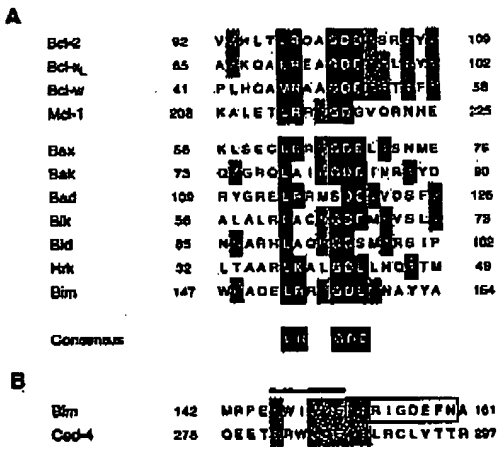

Fig. 9. BH3 homology regions in the Bcl-2 family. (A) Amino acid sequences of the human proteins were aligned with the modified method of Feng and Doolittle (1987) used by the GCG 'PILEUP' program. Residues that are identical or very similar (K and R, D and E, V and I, and M and L) in more than eight of the 11 proteins are shaded in dark grey, while less conserved residues (present in >5/11 proteins) are shaded in light grey. (B) Short stretch of amino acid homology between Bim and *C.elegans* CED-4; this region overlaps with the BH3 region of Bim, indicated by the box. The Bim residues deleted to destroy the CED-4 homology are shown by the solid line.

formed by the BH1, BH2 and BH3 regions of pro-survival molecules such as Bcl-x_L (Sattler *et al.*, 1997). Curiously, however, no residues within the BH3 region clearly distinguish the pro- and anti-apoptotic sets (Figure 9A). Presumably the BH3 regions of the latter are held in a different conformation.

It should be stressed that the four BH3-only proteins are otherwise unrelated and therefore are likely to be regulated by distinct upstream signals. They may also have somewhat different effector functions. For example, neither Bik (Han *et al.*, 1996) nor Bim (Figure 7E) binds to Bax, whereas Bid does—indeed mutagenesis of Bid linked its pro-apoptotic activity more closely to association with Bax than with Bcl-2 (Wang *et al.*, 1996). Another notable difference is that Bid lacks the hydrophobic C-terminal domain found in Bim, Bik and Hrk. As found for several other Bcl-2 family members, that domain probably features in the localization of Bim to intracellular membranes (Figure 3).

To date, Bim is th only BH3-only protein f r which splice variants have been described (Figure 1). All three isoforms interacted *in vivo* with Bcl-2 and Bcl-x_L, but cell death and colony-forming assays revealed clear differences in their cytotoxicity. Since the short form was the most potent inducer of cell death (Figure 7) and suppressor of colony formation (Table I), the regions specific to Bim_L and Bim_{EL} may have a negative regulatory role. Whether the Bim isoforms are expressed differentially in different tissues or developmental stages or in response to particular apoptotic stimuli is currently under study.

Bim can interact with Bcl-2, Bcl-x_L, Bcl-w (Figures 4 and 7) and also with two more distantly related family members that promote cell survival, Mcl-1 (Kozopas *et al.*, 1993) and A1 (Lin *et al.*, 1993) (data not shown). It did not, however, interact with two viral homologues of Bcl-2, adenovirus E1B19K and EBV BHRF-1. In this regard, Bim differs significantly from at least one other BH3-only protein, since Bik/Nbk was isolated by virtue of its binding to E1B19K (Boyd *et al.*, 1995; Han *et al.*, 1996). Unlike Bcl-2, Bcl-x_L and Bcl-w, E1B19K was also an ineffectual antagonist of Bim-induced cell death (Figure 7D). These findings point towards some specificity of interaction between pro- and anti-survival members of the family. Such preferential pairings, where each partner could be regulated independently, may fine-tune the response to multiple survival or death signals. Consistent with this notion, the expression of various pro-survival mediators is induced by distinct signals: Mcl-1 and A1 by myeloid growth factors (Kozopas *et al.*, 1993; Lin *et al.*, 1993), Bcl-2 by IL-7 (von Freeden-Jeffry *et al.*, 1997) and Bcl-x_L by activation of co-receptors such as CD28 (Boise *et al.*, 1995). Regulation of the pro-apoptotic family members is less well studied, but Bax expression can be induced by genotoxic stress via the tumour suppressor p53 (Miyashita *et al.*, 1994). While the levels of Bim mRNA did not change in a T-cell line induced to undergo

apoptosis by two stimuli (Figure 2), it may be induced in other cell types or with different cytotoxic insults.

How might binding of a BH3-containing protein to Bcl-2 trigger apoptosis? In *Caenorhabditis elegans*, the Bcl-2 homologue CED-9 is thought to act by restraining the activity of CED-4, which is required for activation of the caspase CED-3 (Hengartner and Horvitz, 1994). Recent evidence that CED-9, CED-4 and CED-3 associate directly (Chinnaiyan *et al.*, 1997; James *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997), and that Bcl-x_L can bind to CED-4, has raised the possibility that the pro-survival Bcl-2 family members function by sequestering CED-4-like molecules, precluding activation of the caspases (Chinnaiyan *et al.*, 1997; Seshagiri and Miller, 1997; Wu *et al.*, 1997). If this is so, the pro-apoptotic Bcl-2 family members may function by binding to pro-survival relatives and displacing CED-4-like proteins. The recent identification of the first mammalian CED-4 homologue, Apaf-1 (Zou *et al.*, 1997), is expected to facilitate tests of such models. It may be relevant that Bim exhibits a small but striking region of similarity to CED-4 (Figure 9B); in a nine amino acid region partially overlapping BH3, seven (in human Bim) or eight residues (in mouse Bim) are identical to CED-4. A loss-of-function missense mutation in this region of CED-4 (Yuan and Horvitz, 1992) suggested that it might be important for CED-4 function. However, a deletion in Bim that destroyed the homology (Figure 9B) did not interfere with Bim function (not shown). Moreover, the region is not conserved in Apaf-1 (Zou *et al.*, 1997), so the significance of this similarity is unclear.

An alternative hypothesis accounting for the apoptotic activity of BH3-containing proteins is that they directly induce cell death unless complexed by their pro-survival relatives. The inability of E1B19K or Bcl-x_L mutants mt 7 or mt 15 to protect against Bim-induced death is consistent with this hypothesis. Furthermore, genetic experiments have shown that Bax can induce cell death in the absence of Bcl-x_L or Bcl-2 (Simonian *et al.*, 1996; Knudson and Korsmeyer, 1997) and seemingly in the absence of caspase activation (Xiang *et al.*, 1996). The ability of a mutant Bim lacking the BH3 region to suppress colony formation (Table I), albeit not as potently as the wild-type protein, may indicate that Bim retains some pro-apoptotic activity independent of its interaction with other Bcl-2 family members. We currently are testing this hypothesis.

Materials and methods

Expression library screening and isolation of mouse and human bim cDNAs

Polyadenylated RNA prepared from p53$^{-/-}$ KO52DA20 T-lymphoma (Strasser *et al.*, 1994) cells subjected to γ-irradiation (10 Gy) was reverse-transcribed, using a combination of oligo(dT) and random oligonucleotide primers, and ligated to *Eco*RI adaptors using standard procedures. The cDNA was then ligated with *Eco*RI–*Xho*I-digested λ ZapExpress (Stratagene) arms and packaged *in vitro* according to the supplier's instructions. The resulting expression library was screened using radiolabelled Bcl-2 lacking the hydrophobic membrane localization region. To prepare this probe, cDNA encoding amino acids 1–210 of human Bcl-2 was subcloned into the vector pARAR1 (Blanar and Rutter, 1992), and recombinant protein (FLAG-HMK-Bcl-2ΔC30) produced in isopropyl-β-D-thiogalactopyranoside (IPTG)-induced *Escherichia coli* BL21pLysS (DE3) cells (Novagen) was purified on anti-FLAG M2 affinity gel (IBI Kodak) and then kinased *in vitro* using bovine heart muscle kinase (Sigma) and [γ-^{32}P]ATP (Amersham) (Blanar and Rutter, 1992). Approximately 10^6 plaques were screened with ~10^7 c.p.m. of the radiolabelled probe using the protocol of Blanar and Rutter (1992). To reduce non-specific background, lysates from induced parental BL21pLysS (DE3) cells and excess unlabelled ATP were included with the probe. Plaques that were positive on duplicate lifts were picked for two rounds of further screening. Positive clones were excised *in vivo* by co-infection with filamentous ExAssist (Stratagene) helper phage and sequenced by automated sequencing (ABI Perkin Elmer).

The human *bim* cDNA clone was isolated by screening human fetal spleen (Stratagene) and peripheral blood leukocyte (Clontech) λ cDNA libraries with an ~800 bp mouse *bim* cDNA probe, using standard techniques. The cDNAs were fully sequenced, analyzed using GCG or DNASTAR software and compared with sequences in the GenBank (including dbEST) and EMBL databases using the BLAST algorithm (Altschul *et al.*, 1990).

Expression constructs and site-directed mutagenesis

cDNAs were cloned into the expression vectors pEF PGKpuro (Huang *et al.*, 1997a) or pEF PGKhygro (Huang *et al.*, 1997a), or derivatives of these incorporating N-terminal FLAG (DYKDDDDK) (Hopp *et al.*, 1988) or EE (EYMPME) (Grussenmeyer *et al.*, 1985) epitope tags. The *bim*ΔBH3 mutation was generated by deleting the DNA encoding amino acids 94–100 (LRRIGDE) and replacing this with DNA corresponding to a *Hin*dIII site (encoding AL). Mutations in *bcl*-2 (ΔBH4, G145E, W188A) (O'Reilly *et al.*, 1996) were generated by PCR via splice overlap extension (Horton *et al.*, 1993) using the proof-reading *Pfu* DNA polymerase (Stratagene) (details of oligonucleotides used will be supplied on request). The sequences of derived clones were verified by automated sequencing.

Cell culture and transfection

Cell lines used were: mouse IL-3-dependent promyelocytic line FDC-P1, mouse T-hybridoma B6.2.16BW2, mouse B-lymphoma lines CH1 and WEHI 231, mouse pre-B lymphoma line WEHI 415 (derived from a tumour which arose in an Eμ-*myc* transgenic mouse), human B-lymphoblastoid line SKW6, human T-lymphoma line Jurkat, mouse T-lymphoma lines WEHI 703 and WEHI 707 (both derived from tumours which arose in Eμ-N-Ras transgenic mice) and WEHI 7.1, rat fibroblastoid line Rat-1, mouse fibroblastoid line NIH-3T3, mouse fibroblastoid line L929 subline LM(-TK), human embryonal kidney cell line 293 (ATCC CRL-1573) and SV40-transformed 293 cells, 293T (see Lithgow *et al.*, 1994; Strasser *et al.*, 1994, 1995; Huang *et al.*, 1997a). The procedures for culture and stable transfection are described elsewhere (Strasser *et al.*, 1995; Huang *et al.*, 1997a,b). Drug-resistant transfectants were cloned using the cell deposition unit of a FACStarPlus (Becton Dickinson) and clones expressing high levels of the protein of interest were identified by immunofluorescence staining of fixed and permeabilized cells followed by flow cytometric analysis.

Cell death assays

Cytokine deprivation, exposure to ionizing radiation and treatment with staurosporine (Sigma) were the principal cell death assays used to assess the sensitivity of FDC-P1 cells stably transfected with the various expression vectors. Cells were cultured in medium lacking cytokine or (in complete medium) after exposure to 10 Gy γ-radiation (provided by a ^{60}Co source at 3 Gy/min) and their viability determined over several days by vital dye (0.4% eosin) exclusion, as assessed by visual inspection in a haemocytometer, or by flow cytometric analysis of cells that excluded propidium iodide (5 μg/ml, Sigma).

Cell death assays in 293T cells were performed after transient transfection of ~5×10^5 cells using 6 μl of Lipofectamine® (Gibco BRL) and a total of 1 μg of DNA in 2 ml of medium in 6 cm dishes; for co-transfections, *bim* plasmid (0.1, 0.2 and 0.5 μg) was co-transfected with 0.5 μg of the other recombinant (e.g. *bcl*-2) plasmid plus 0.4 or 0.3 μg or none of the empty vector. At 48 h after transfection, the cells were harvested, fixed for 5 min in 80% methanol, permeabilized with 0.3% saponin (which was reduced to 0.03% in all the subsequent steps) and stained with 1 μg/ml anti-EE monoclonal antibody (BabCO), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1 μg/ml, Southern Biotechnology) as the secondary agent and by 69 μM propidium iodide in 38 mM sodium citrate pH 7.4 containing 5 μg/ml RNase A (Huang *et al.*, 1997b). Analysis was performed on a FACScan II (Becton Dickinson), the proportion of dead cells being taken as the proportion of EE-positive cells with less than 2C DNA content (Nicoletti *et al.*, 1991).

L929 fibroblast colony assays were performed in triplicate by scoring

the numbers of colonies in 10 cm dishes grown for 14–18 days with appropriate antibiotic selection. These cells previously had been split (1:3) from ~10^6 cells which had been transfected in 6 cm dishes 2 days earlier with 1 μg total DNA and 12 μl of Lipofectamine®.

Immunofluorescence, subcellular fractionation, immunoprecipitation and immunoblotting

Immunofluorescence staining of cytoplasmic proteins with the monoclonal antibodies Bcl-2-100 [mouse anti-human Bcl-2 (Pezzella *et al.*, 1990)] or mouse anti-EE (BabCO) followed by FITC-conjugated goat anti-mouse IgG (Southern Biotechnology) was performed as previously described (Strasser *et al.*, 1995; Huang *et al.*, 1997a). Cells were analysed using a FACScan (Becton Dickinson) after exclusion of dead cells on the basis of their forward and side scatter characteristics.

To investigate the subcellular localization of EE-tagged Bim_L, L929 fibroblasts transfected by lipofection (see above) were grown in chamber slides (Erie Scientific Company, New Hampshire), fixed in 4% paraformaldehyde for 10 min at room temperature and the slides were then allowed to dry and stored at −20°C. Prior to analysis by confocal microscopy, the cells were rehydrated and then permeabilized for 15 min at room temperature in 0.5% Triton-X 100 in phosphate-buffered saline (PBS). EE-Bim_L was detected by incubating the cells with anti-EE monoclonal antibody for 30 min, washing several times in PBS containing 1% fetal calf serum and 0.05% Tween-20, and then incubating for 30 min with either FITC-conjugated goat anti-mouse IgG (Southern Biotechnology) or lissamine–rhodamine-conjugated goat anti-mouse IgG (Jackson Immunoresearch); all steps were performed at room temperature. Human Bcl-2 was detected similarly, using hamster anti-human Bcl-2 (6C8) (Veis *et al.*, 1993) followed by FITC-conjugated mouse anti-hamster IgG (Pharmingen). Untransfected cells served as negative controls. Samples were analyzed with a Leica confocal laser scanning microscope using SCANware software (Leica Lasertechnik, Heidelberg, Germany).

For subcellular fractionation, lysates were made with a Dounce homogenizer (Hsu *et al.*, 1997) from ~10^6 cells in lysis buffer: 10 mM Tris–HCl pH 7.4, 0.5 μg/ml Pefabloc, 1 μg/ml each of leupeptin, aprotinin, soybean trypsin inhibitor and pepstatin, 5 mM NaF and 2 mM Na_3VO_4 (Sigma or Boehringer Mannheim). The lysates were centrifuged at 900 *g* for 10 min to obtain the nuclear pellet and the supernatant centrifuged at 130 000 *g* for 60 min to obtain the membrane fraction.

To test for protein–protein interactions *in vivo*, co-immunoprecipitation followed by immunoblotting was performed on stably transfected FDC-P1 cells or transiently transfected 293T cells as described previously (Huang *et al.*, 1997a,b). Briefly, lysates prepared from 10^5–10^6 cells were incubated with ~5 μg of antibody (anti-human Bcl-2, anti-FLAG M2 (IBI Kodak) or anti-EE monoclonal antibody), followed by protein G–Sepharose (Pharmacia), and then pelleted, washed, fractionated by SDS–PAGE and transferred to nitrocellulose membranes by electroblotting. The filters were incubated with mouse anti-human Bcl-2, anti-FLAG or anti-EE antibodies followed by affinity-purified rabbit anti-mouse IgG (Jackson Immunoresearch). Bound antibodies were detected with ^{125}I-labelled staphylococcal protein A, except in the subcellular fractionation experiments, where bound antibodies were revealed with horseradish peroxidase-conjugated sheep anti-mouse Ig (Silenus) and enhanced chemiluminescence (Amersham). In some experiments, the cells were metabolically labelled with 100–200 μCi/ml of [^{35}S]methionine (NEG-072 from NEN), and equivalent trichloroacetic acid-precipitable counts (5×10^7 c.p.m.) were used for each immunoprecipitation.

Accession numbers

The sequences described here have been deposited with GenBank, the accession numbers are: human bim_{EL} (AF032457), human bim_L (AF032458), mouse bim_{EL} (AF032459), mouse bim_L (AF032460) and mouse bim_S (AF032461).

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The Proapoptotic Activity of the Bcl-2 Family Member Bim Is Regulated by Interaction with the Dynein Motor Complex

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Summary

Bcl-2 family members that have only a single Bcl-2 homology domain, BH3, are potent inducers of apoptosis, and some appear to play a critical role in developmentally programmed cell death. We examined the regulation of the proapoptotic activity of the BH3-only protein Bim. In healthy cells, most Bim molecules were bound to LC8 cytoplasmic dynein light chain and thereby sequestered to the microtubule-associated dynein motor complex. Certain apoptotic stimuli disrupted the interaction between LC8 and the dynein motor complex. This freed Bim to translocate together with LC8 to Bcl-2 and to neutralize its antiapoptotic activity. This process did not require caspase activity and therefore constitutes an initiating event in apoptosis signaling.

Introduction

Apoptosis signaling pathways converge upon a common death effector machinery (Vaux and Strasser, 1996). At least three classes of proteins are involved: cysteine proteases (caspases), their adaptor proteins, and proapoptotic members of the Bcl-2 family. Cysteine proteases, such as the 14 known mammalian caspases and *Caenorhabditis elegans* CED-3, cleave substrates at aspartate residues (Thornberry and Lazebnik, 1998). In healthy cells, caspases are found as zymogens that must be cleaved at specific aspartate residues so that the prodomain and two polypeptides of ~20 kDa and ~10 kDa are separated to facilitate assembly of the active tetrameric ($p20_2p10_2$) enzyme. Apoptosis seems to proceed by initiation of a caspase cascade. Caspases with long prodomains are the first to be activated, and this is thought to occur by self-processing (Martin et al., 1998; Muzio et al., 1998; Srinivasula et al., 1998). These "initiator caspases" process so-called "effector caspases," which in turn cleave a number of vital cellular proteins and activate latent enzymes that execute terminal events in apoptosis (Liu et al., 1997; Enari et al., 1998). Adaptor proteins, such as mammalian Apaf-1 and *C. elegans* CED-4, promote self-processing of initiator caspases by aggregating their zymogens (Seshagiri and Miller, 1997; Zou et al., 1997; Srinivasula et al., 1998; Yang et al., 1998).

The antiapoptotic members of the Bcl-2 family—mammalian Bcl-2, Bcl-x_L, Bcl-w, A1/Bfl-1, Mcl-1, and Boo as well as *C. elegans* CED9—contain 3–4 domains with sequence homology (Adams and Cory, 1998). These Bcl-2 homology regions (BH1–4) mediate protein-protein interactions and are essential for survival function (Oltvai and Korsmeyer, 1994). Genetic and biochemical analyses indicated that antiapoptotic Bcl-2 family members inhibit cell death by keeping adaptor proteins in check (Shaham and Horvitz, 1996; Chinnalyan et al., 1997; Spector et al., 1997; Wu et al., 1997). The proapoptotic members of the Bcl-2 family, particularly those that have only a BH3 domain but are otherwise unique, induce cell death by binding the antiapoptotic Bcl-2 family members and neutralizing their effect on adaptor proteins. The BH3-only subfamily presently includes mammalian Bad, Blk/Nbk, Bid, Hrk/DP5, Bim, Bik, and *C. elegans* EGL-1 (Adams and Cory, 1998). The following model can be inferred from these results. In healthy cells, antiapoptotic Bcl-2 family members prevent self-processing of "initiator caspases" by blocking the action of adaptor proteins. Apoptotic stimuli induce the activity of proapoptotic Bcl-2 family members that bind to the antiapoptotic Bcl-2 family members. This frees adaptor proteins to initiate the caspase cascade.

By screening a cDNA expression library using recombinant Bcl-2 as bait, we isolated the BH3-only protein Bim (O'Connor et al., 1998). Bim contains a hydrophobic C-terminal region, and upon transient overexpression it localizes to the cytoplasm. Alternative splicing gives rise to three isoforms: Bim_S, Bim_L, and Bim_{EL} (O'Connor et al., 1998). All three isoforms are potent inducers of apoptosis and can only be expressed stably in cultured cell lines if Bcl-2 or a functional homolog is coexpressed at high levels (O'Connor et al., 1998). The BH3 region is essential for the proapoptotic activity of Bim, although a mutant lacking this domain can slightly suppress colony formation of L929 cells by nonapoptotic mechanisms. Of the three isoforms, Bim_S is considerably more cytotoxic than Bim_L or Bim_{EL}, indicating that the additional regions present in the longer forms may attenuate proapoptotic activity (O'Connor et al., 1998). Bim_L and Bim_{EL} are the predominant isoforms expressed in cell lines and normal tissues (O'Reilly et al., 1998).

Here we describe the regulation of the proapoptotic activity of Bim. Experiments using the yeast two-hybrid system and coimmunoprecipitation assays demonstrated that Bim_L and Bim_{EL}, but not Bim_S, bound to LC8, a component of the microtubule-associated dynein motor complex. This interaction has physiological significance, since single amino acid substitutions in Bim_L that abolished this interaction enhanced its proapoptotic activity. In healthy cells, endogenous Bim_L was bound to LC8 and sequestered to the microtubule-associated dynein motor complex. Certain apoptotic stimuli caused a complex of Bim_L and LC8 to dissociate from the dynein motor complex and translocate to Bcl-2. This process was not merely a consequence of cell death but occurred

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independently of caspase activation and therefore constitutes an upstream event in apoptosis signaling.

Results

Yeast Two-Hybrid Library Screening with Bim_L

Screening of bone marrow and brain cDNA libraries (1.5 million clones of each) with mouse Bim_L as the bait yielded 15 clones encoding several of the known anti-apoptotic Bcl-2 family members: Bcl-2, Bcl-x_L, A1/Bfl-1, and Mcl-1. These proteins bind to Bim_L with high affinity (O'Connor et al., 1998), providing evidence that the screening worked efficiently and with a high degree of specificity. Screening of a HeLa cell library (3.5 million clones) yielded 52 clones encoding LC8, also called cytoplasmic dynein light chain or protein inhibitor of neuronal nitric oxide synthase (Jaffrey and Snyder, 1996; King et al., 1996). Nucleotide sequence analysis of 20 LC8 clones showed that they fell into four groups that varied in their 5′-untranslated regions. Clones from three of these groups had stop codons in all three reading frames upstream of the start codon. The fourth group contained full-length LC8 fused in-frame with the GAL4 activation domain. Examination of sequences from the first three clones revealed consensus splice acceptor sites upstream of the coding region in these LC8 clones. The coding region of LC8 alone, but not the 5′-untranslated region, when fused to the GAL4 activation domain interacted with Bim_L fused to the GAL4 DNA-binding domain (Figure 1A).

In the yeast two-hybrid assay, LC8 interacted with Bim_L and Bim_{EL} but not with Bim_S, and/or with ten irrelevant baits—Bax, c-Jun, CD4, PEA-15, SNF4, NGFR, FADD/MORT1, baculovirus p35, caspase-2, and caspase-8 (Figure 1A and data not shown). These results show that Bim_L can interact specifically with LC8 and that this requires the region within Bim_L that is absent in Bim_S.

Bim_L and Bim_{EL} Bind Specifically to LC8 in Mammalian Cells

To see if Bim_L and Bim_{EL} could interact with LC8 in cells, we performed coimmunoprecipitation experiments. When transiently coexpressed in the human embryonic kidney cell line 293 T, interaction between EE epitope-tagged Bim_L and FLAG epitope-tagged LC8 was readily detected (Figure 1B). Consistent with the results obtained from the yeast two-hybrid system, Bim_L and Bim_{EL}, but not Bim_S, were able to bind to LC8 (Figures 1B and 1C). Deletion of the BH3 domain had no effect on the ability of Bim_L to interact with LC8 (Figures 1A and 1C) but abolished its binding to Bcl-2 or its homologs (O'Connor et al., 1998). Other proapoptotic members of the Bcl-2 family, such as Bak, Bax, Bad, Bik/Nbk, or Bid, and the adaptor proteins FADD/MORT1 or TRADD, did not coimmunoprecipitate with LC8 (Figure 1C and data not shown).

The interaction between Bim_L and LC8 was not merely an artifact of transient overexpression. In IL-3-dependent FDC-P1 cells, stably transfected with EE-Bim_L and Bcl-2 expression constructs, endogenous LC8 could be coimmunoprecipitated with Bim_L (Figure 1D). A tripartite complex between Bcl-2, Bim_L, and endogenous LC8 was detected in which Bim_L appeared to be the bridging molecule because LC8 could only be coimmunoprecipitated by anti-Bcl-2 antibodies in cells expressing Bim_L (Figure 1D). Similarly, in 293 T cells transiently cotransfected with EE-Bim_L and Bcl-x_L expression constructs, immunoprecipitates of Bcl-x_L contained Bim_L and endogenous LC8 (Figure 1E). The relative strengths of the [^{35}S]methionine signals of Bim_L and LC8 in these experiments indicated that the interaction was stoichiometric at 1:1 (Figures 1D and 1E). Most importantly, in the human breast carcinoma line MCF-7, endogenous LC8 could be coimmunoprecipitated with endogenous Bim_L (Figure 1F). These results confirm that Bim_L and Bim_{EL} can interact specifically with LC8 and provide compelling evidence that this interaction occurs under physiological conditions.

Amino Acids 51–54 of Bim_L Are Required for Binding to LC8

LC8 was likely to interact with the region between amino acids 42–71 of Bim_L since Bim_S lacks this region and does not bind to LC8 (Figure 2A). To determine the precise residues of Bim_L required for LC8 binding, a fine mapping approach was undertaken using the yeast reverse two-hybrid system (Vidal et al., 1996). In this assay, binding of Bim_L (coupled to the GAL4 DNA-binding domain) to LC8 (fused to the GAL4 activation domain) induces expression of orotidine-5-phosphate decarboxylase, which converts 5-fluoroorotic acid to 5-fluoro-uracil. Since 5-FU is cytotoxic, a positive interaction results in abrogation of yeast growth. The region within Bim_L spanning amino acids 1–86 was mutagenized by low-fidelity PCR, and the resulting cDNAs recombined into the yeast vector encoding Bim_L. From 15,000 transformants screened, 82 clones of Bim_L that did not interact with LC8 were isolated. Clones encoding truncated Bim_L were eliminated by their inability to interact with Bcl-2. This was feasible because the BH3 region of Bim_L, necessary for its interaction with Bcl-2, is C-terminal to the putative LC8-binding region and would therefore be lost in such truncation mutants (Figure 2B). This led to the identification of 24 Bim_L clones that did not interact with LC8 but could bind Bcl-2. Sequence analysis revealed that all clones contained substitutions within amino acid residues 42–71 of Bim_L, particularly between amino acids 51 and 54 (Figure 2B).

Four of the Bim_L mutants recovered from this screen were chosen for detailed investigation: D51G, S53P, T54A, and the double mutant T54A/N65S. A quantitative analysis of the strength of interaction between LC8 and wt or mutant Bim_L was performed in a yeast two-hybrid assay by measuring β-galactosidase activity using o-nitrophenyl β-D-galactopyranoside as the substrate. The S53P, T54A, and T54A/N65S Bim_L mutants had less than 0.1% of the LC8 binding activity of wild-type Bim_L while the D51G Bim_L mutant had ~5% of wt activity (Figure 2C).

Interactions of wild-type Bim_L or the four Bim_L mutants with LC8 or Bcl-2 wer also studied in coimmunoprecipitation assays with FDC-P1 cells stably expressing Bcl-2 and various forms of Bim_L. All four Bim_L mutants interacted efficiently with Bcl-2 but did not bind to endogenous LC8 (Figure 2D). The different results obtained with

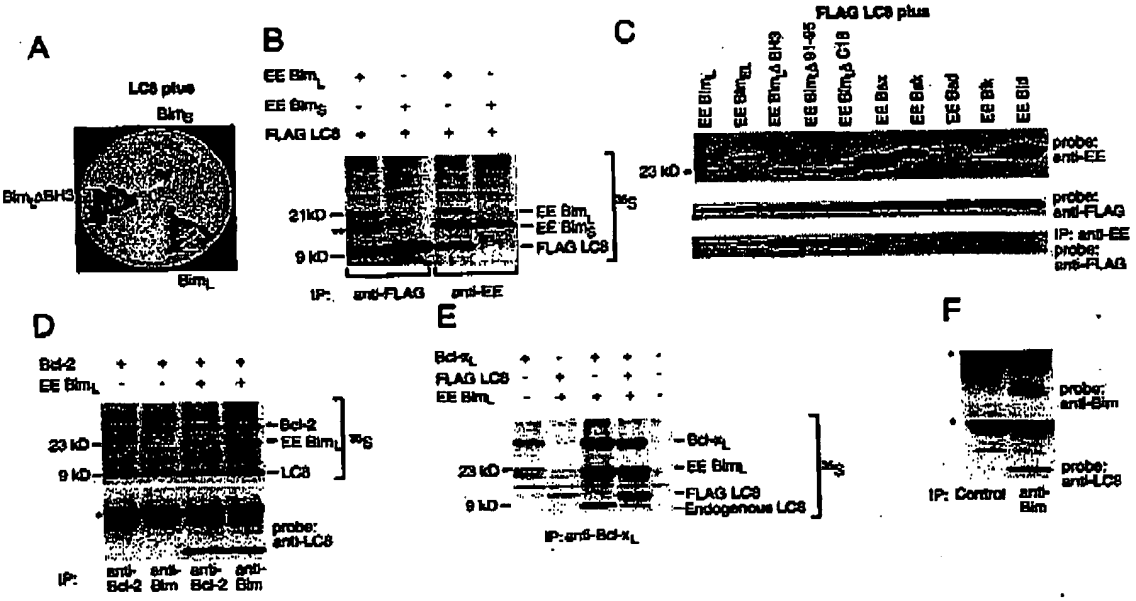

Figure 1. Bim$_L$ and Bim$_{EL}$, but Not Bim$_S$, Bind to LC8

(A) Yeast cells were cotransformed with an expression construct encoding LC8 coupled to the GAL4 activation domain and constructs encoding a fusion protein containing the GAL4 DNA-binding region fused to Bim$_L$, Bim$_L$ mutant lacking the BH3 domain, or to Bim$_S$. Cells were grown on selective medium and assayed for β-galactosidase activity.

(B) Lysates from [^{35}S]methionine-labeled 293 T cells coexpressing FLAG-LC8 and either EE-Bim$_L$ or EE-Bim$_S$ were immunoprecipitated with anti-FLAG or anti-EE antibodies. Double asterisk, bands in lanes 1 and 3 are breakdown products of EE-Bim$_L$.

(C) 293 T cells were transiently cotransfected with expression constructs encoding FLAG-LC8 and EE-tagged proapoptotic proteins. The upper two panels represent Western blots documenting protein expression. The bottom panel shows the coimmunoprecipitation analysis. Lysates were precipitated with anti-EE antibody, and Western blotting was performed with anti-FLAG antibody.

(D) The upper panel shows metabolically labeled lysates from FDC-P1/Bcl-2 (lanes 1 and 2) or FDC-P1/Bcl-2/EE-Bim$_L$ (lanes 3 and 4) immunoprecipitated with anti-Bcl-2 (lanes 1 and 3) or anti-Bim antibodies (lanes 2 and 4). The lower panel is the same blot stained with anti-LC8 antibody to detect endogenous LC8.

(E) Lysates from metabolically labeled 293 T cells cotransfected with expression constructs encoding Bcl-x$_L$, EE-Bim$_L$, and FLAG-LC8 either alone or in combinations were immunoprecipitated with anti-Bcl-x antibody.

(F) Lysates from MCF-7 cells were immunoprecipitated with anti-Bim antibody. Western blotting was performed with anti-Bim (top panel) or anti-LC8 antibodies (bottom panel). Asterisk, IgL chain from the antibody used for IP.

the D51G Bim$_L$ mutant in the yeast two-hybrid system and the coimmunoprecipitation analysis reflect the greater sensitivity of the former technique, which provides reliable quantitation. These results demonstrate that amino acids 51–54 of Bim$_L$ are critical for its binding to LC8.

Mapping of the Bim-Binding Region in LC8

LC8 is known to homodimerize, and this interaction requires an N-terminal region spanning amino acid residues 10–28 (Benashski et al., 1997). To identify the Bim-binding region in LC8, we performed coimmunoprecipitation experiments with extracts from 293 T cells transiently cotransfected with expression constructs encoding Bim$_L$ and wt or deletion mutants of LC8. Bim$_L$ bound to wt LC8 and to truncated LC8 lacking the N-terminal 15 or 30 amino acids. In contrast, Bim$_L$ did not bind to a mutant of LC8 missing the C-terminal 15 amino acids (Figure 2E). These results show that LC8 has distinct regions for binding to Bim and for homodimerization. It is therefore possible that Bim$_L$ and Bim$_{EL}$ bind to LC8 homodimers.

Binding to LC8 Regulates the Proapoptotic Activity of Bim

LC8 is a component of the minus end–directed dynein motor complex, an evolutionarily conserved microtubule-bound ATPase involved in flagellar movement in *Chlamydomonas* and in retrograde organelle transport in mammalian cells (Hirokawa, 1998). Dynein heavy chains and dynein intermediate chains are integral structural components of the dynein ATPase complex. LC8 is also a component of this complex but its biochemical function is unknown (King et al., 1996).

Bim$_S$, the splice variant of Bim, which does not bind to LC8 (Figures 1A and 1B), is a more potent inducer of cell death than Bim$_L$ or Bim$_{EL}$ (O'Connor et al., 1998). We therefore speculated that binding to LC8 might regulate the proapoptotic activity of Bim by sequestering it to the microtubule-associated dynein motor complex away from Bcl-2 and its homologs. To test this idea, we generated FDC-P1 clones stably expressing Bcl-2 together with Bim$_S$, Bim$_L$, or mutants of Bim$_L$ that did not bind to LC8 and measured their sensitivity to apoptotic stimuli. Four clones of each genotype, matched for equivalent levels of Bcl-2 and Bim, were selected for

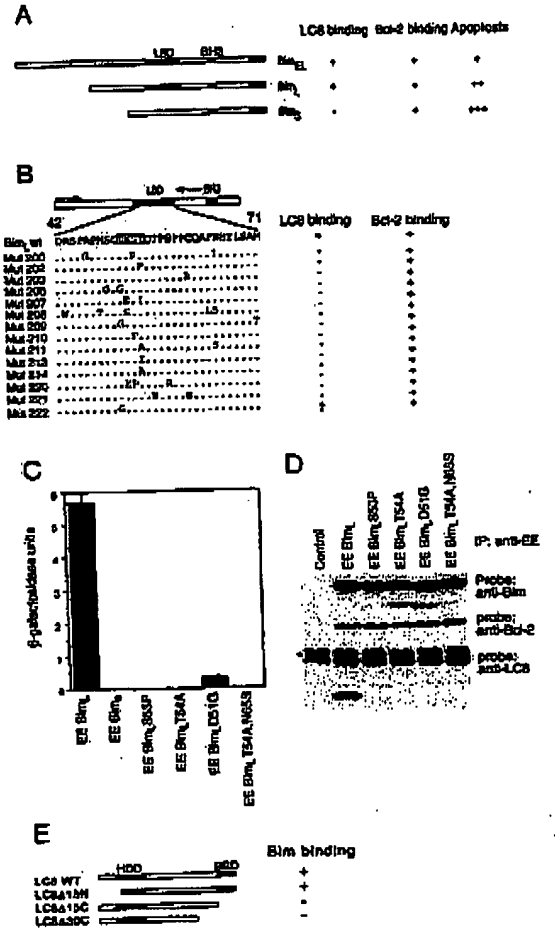

Figure 2. Mapping of the LC8-Binding Region in Bim_L and the Bim-Binding Region in LC8

(A) Structure of the three Bim isoforms with the putative LC8-binding region (LBD), the BH3 domain, and the hydrophobic C-terminal region (hatched boxes). The ability of the isoforms to bind to LC8 and Bcl-2 and their proapoptotic activity are indicated on the right.

(B) The location of the primers is shown on top, and sequences of the mutant Bim_L alleles recovered are shown at the bottom. The ability of the Bim_L mutants to bind to Bcl-2 or LC8 and their proapoptotic activity are indicated on the right.

(C) The relative affinity of interaction between wt or mutant Bim_L and LC8 was determined in a yeast two-hybrid assay by measuring β-galactosidase activity.

(D) The ability of wt or mutant Bim_L to bind to Bcl-2 or LC8 was tested in stably transfected FDC-P1 cells. Lysates from FDC-P1 cells expressing Bcl-2 alone (control) or coexpressing Bcl-2 plus wt EE-Bim_L or EE-Bim_L mutants were immunoprecipitated with anti-EE antibody. The top panel shows the blot probed with anti-Bim antibody documenting the expression of EE-Bim_L. The middle panel shows the blot probed with anti-Bcl-2 antibody. The bottom panel shows the blot probed with anti-LC8 antibody to detect endogenous LC8. Asterisk, IgL chain from the antibody used for IP.

(E) The Bim-binding region in LC8 was determined by coimmunoprecipitation of proteins from lysates of 293 T cells cotransfected with expression constructs encoding EE-Bim_L plus FLAG-tagged wild-type or truncation mutants of LC8. The homodimerization (HDD) and the Bim-binding domains (BBD) are indicated.

analysis (Figures 2D and 3A). Parental FDC-P1 cells and transfectants expressing only Bcl-2 were used as controls. Upon cytokine deprivation or γ irradiation, FDC-P1 cells expressing Bcl-2 and Bim_S died much more rapidly than those expressing Bcl-2 and Bim_L (Figure 3B). The three Bim_L mutants that cannot interact with LC8 (S53P, T54A, and T54A/N65S) were as potent as Bim_S (Figure 3B). Differences in cell survival between clones expressing Bcl-2 plus wt Bim_L and those expressing Bcl-2 plus wt Bim_S or Bcl-2 plus mutant Bim_L ranged between 2.5- to 5-fold. In contrast, the D51G Bim_L mutant, which retains detectable binding to LC8, did not have significantly altered killing potential and behaved like wt Bim_L (Figure 3B).

Consistent with these results, transfection with expression constructs encoding Bim_S or the Bim_L mutants S53P, T54A, and T54A/N65S suppressed colony formation of L929 cells more potently than did transfection with wt Bim_L or mutant Bim_L D51G expression constructs (Figure 3C). These results demonstrate that interaction with LC8 can regulate the proapoptotic activity of Bim, presumably by delaying its access to antiapoptotic Bcl-2 family members in cells exposed to a death stimulus.

During Induction of Apoptosis, Bim_L and LC8 Are Released Together from the Dynein Motor Complex

A large portion of LC8 molecules are integral components of the microtubule-associated dynein motor complex (King et al., 1996) or the actin-based myosin complex (Benashski et al., 1997). Since Bim_L and Bim_{EL} bind to LC8, we examined the cytoskeletal association of different isoforms and mutants of Bim. Cytoskeleton-associated proteins were separated from the remainder of the cellular proteins on sucrose gradients. Bim associated with microtubules should sediment with a higher coefficient, whereas free Bim should sediment with a lower coefficient. In extracts from FDC-P1 cells coexpressing Bcl-2 and Bim_L, a large portion of Bim_L sedimented with a high coefficient in the same fractions in which endogenous LC8 was found (Figure 4). In contrast, Bim_S and the three mutants of Bim_L that do not bind to LC8 sedimented with a lower sedimentation coefficient (Figure 4). Bim_L D51G, which has detectable affinity for LC8 and unaltered proapoptotic activity, migrated like Bim_L on the gradient (Figure 4). Densitometric analysis confirmed these observations, consistent with the notion that the D51G mutant retained the ability to interact with the dynein motor complex. These results support the idea that the killing potential of Bim_L can be regulated through its interaction with LC8, an integral component of the microtubule-associated dynein motor complex.

The subcellular localization of Bim_L during induction of apoptosis was studied in FDC-P1/Bcl-2/Bim_L cells, which were starved of IL-3 or γ irradiated. After these apoptotic stimuli, the sedimentation of Bim_L on sucrose gradients changed from the heavier fractions, in which microtubule components and th associated dynein motor complex migrated, to the lighter fractions (Figure 5A). The relocation of Bim_L occurred within 12 hr of IL-3

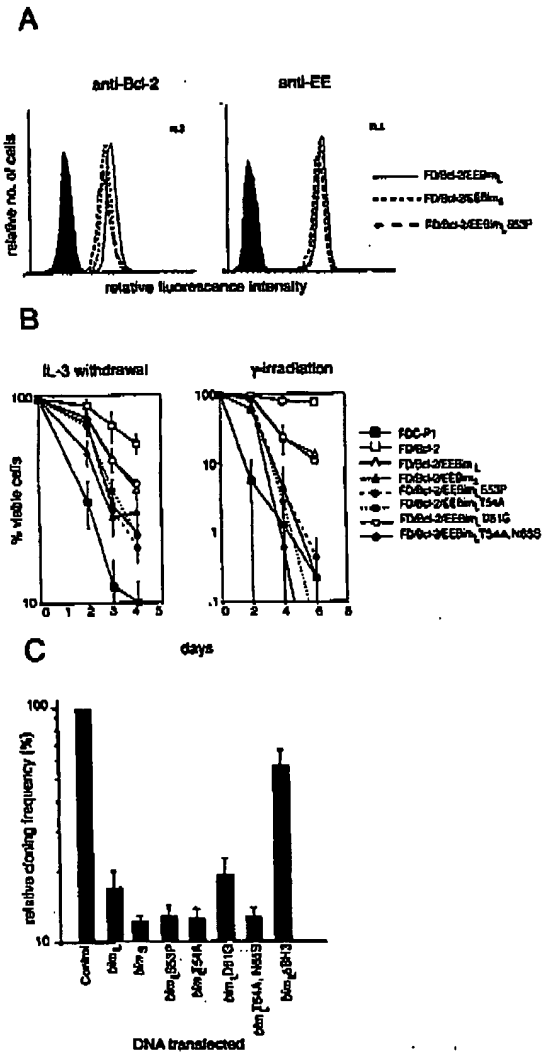

Figure 3. Binding to LC8 Regulates the Proapoptotic Activity of Bim$_L$

(A) Flow cytometric analysis of parental FDC-P1 cells (shaded) and representative clones to document the level of Bcl-2 and EE-Bim$_L$ isoforms or mutants.

(B) Parental FDC-P1 cells, clones expressing Bcl-2 alone, and clones coexpressing Bcl-2 and different EE-Bim isoforms or mutants were deprived of IL-3 (left panel) or γ irradiated with 10 Gy (right panel). Cell survival was determined after 1–6 days by PI staining and flow cytometric analysis. Data shown represent arithmetic means ± SD of three experiments on >3 independent clones of each genotype. Coexpression of Bcl-2 was necessary to produce lines stably expressing Bim because of its potent proapoptotic activity (O'Connor et al., 1998).

(C) Suppression of L929 fibroblast colony formation by Bim isoforms or mutants. Control DNA or constructs encoding EE-Bim isoforms or mutants were cotransfected with a puromycin resistance vector into L929 cells. The numbers of puromycin-resistant colonies after 14 days of selection were scored. Data shown represent arithmetic means ± SD of >3 independent experiments.

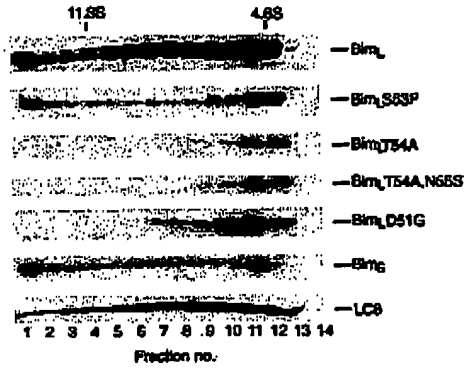

Figure 4. Wild-Type Bim$_L$ Associates with the Dynein Motor Complex

Extracts of FDC-P1 cells stably expressing Bcl-2 and various forms of EE-Bim were fractionated by sucrose gradient sedimentation. Proteins in gradient fractions were size separated by gel electrophoresis and transferred to membranes that were probed with anti-EE antibody or antibody to LC8. The positions of sedimentation markers are indicated: BSA, 4.6S$_{s,w}$; bovine catalase, 11.3S$_{s,w}$.

withdrawal, at a time when >95% of cells were still viable and the microtubules, as judged by staining with antibodies to α-tubulin, remained intact (Figure 5A). The broad spectrum caspase inhibitor zVAD-fmk inhibited cell death but had no effect on Bim$_L$ relocalization, demonstrating that this event occurred during the initiation of apoptosis and was not simply a consequence of caspase activation (Figure 5B).

The sucrose gradient fractionation experiments showed that LC8 was present in the same fractions as Bim$_L$ in extracts of healthy cells and extracts from IL-3-deprived cells primed to undergo apoptosis (Figure 5A). Presumably the two proteins did not comigrate perfectly because LC8 is more abundant than Bim$_L$ and can also associate with proteins other than Bim$_L$. Consistent with the idea that Bim$_L$ and LC8 remained associated during induction of cell death, the two proteins could be coimmunoprecipitated from healthy cells and from cells undergoing apoptosis (Figures 1E and 6). In contrast, when the sedimentation patterns of dynein intermediate chain and α-tubulin were studied, no significant difference was observed between healthy cells and dying cells (Figure 5A). Moreover, in FDC-P1 cells overexpressing Bcl-2 but not Bim$_L$ (FDC-P1/Bcl-2), LC8 was released upon IL-3 withdrawal even though no Bim was present and apoptosis was blocked (Figure 5C). These experiments demonstrate that during induction of apoptosis, even prior to caspase activation, LC8 breaks away from the dynein or myosin V motor complexes. As a result, Bim$_L$ and LC8 are released from the cytoskeletal structures where the motor complexes are localized.

Liberated Bim$_L$–LC8 Complexes Translocate to Bcl-2

T verify our observations on the translocation of Bim$_L$ and LC8 during induction of apoptosis, we studied a cell type in which Bim$_L$ is expressed at physiological levels. The MCF-7 c ll line expresses readily detectable levels of Bcl-2 and Bim$_L$. Apoptosis can be induced in

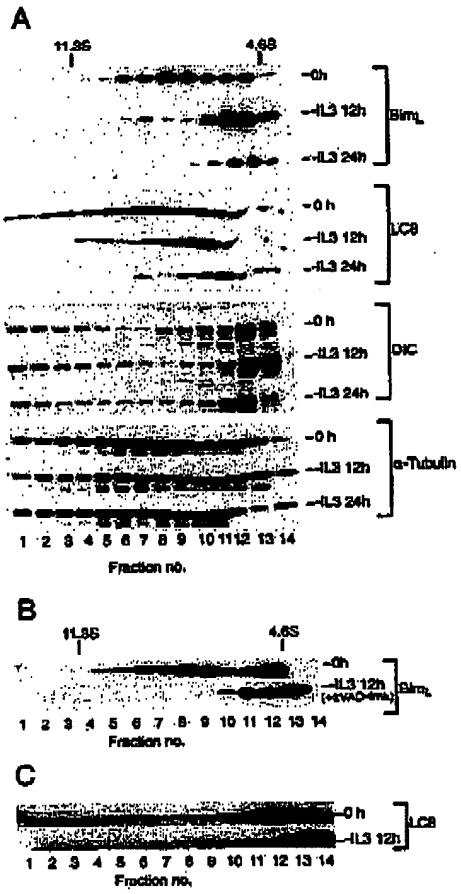

Figure 5. During Induction of Apoptosis, Bim$_L$ and LC8 Dissociate Together from the Dynein Motor Complex

(A) FDC-P1 cells stably expressing Bcl-2 and EE-Bim$_L$ were cultured in the absence of IL-3 for 0, 12, or 24 hr. Extracts were prepared and fractionated by sucrose gradient sedimentation. Proteins in gradient fractions were size separated by gel electrophoresis, transferred to membranes, and probed with antibodies to Bim (panels 1–3), LC8 (panels 4–6), dynein intermediate chain (panels 7–9), or α-tubulin (panels 10–12).

(B) FDC-P1/Bcl-2/Bim$_L$ cells were cultured with IL-3 (upper panel) or for 12 hr without IL-3 in the presence of the caspase inhibitor zVAD-fmk (lower panel). Proteins were extracted, fractionated, and blotted as in (A) and membranes were probed with antibody to Bim.

(C) FDC-P1/Bcl-2 cells were cultured with IL-3 (upper panel) or for 12 hr without IL-3 (lower panel). Proteins were extracted, fractionated, and blotted as in (A), and membranes were probed with antibodies to LC8. The positions of sedimentation markers are indicated. A general decrease in levels of most proteins occurs during induction of apoptosis.

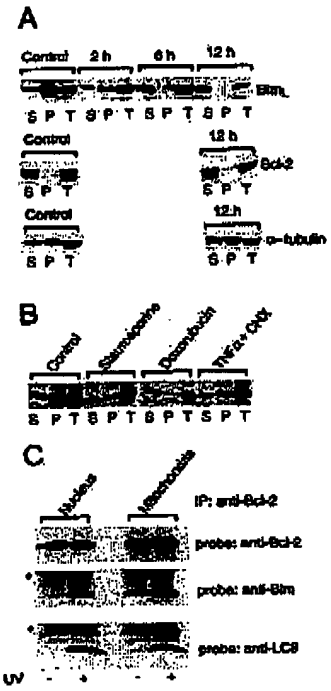

Figure 6. During Induction of Apoptosis, LC8 and Bim$_L$ Translocate Together to Bcl-2

(A) MCF-7 cells were left untreated or were harvested 2, 4, and 12 hr after UV irradiation at 100 J/m^2. Soluble (S) and insoluble (P) fractions were prepared by treatment of cells with taxol to fix cytoskeletal proteins and centrifugation. Total cell extracts (T) served as controls. Proteins were separated by gel electrophoresis, and membranes were probed with antibodies to Bim (upper panel), Bcl-2 (middle panel), or α-tubulin (lower panel).

(B) Total cell extracts (T), soluble (S), or insoluble (P) fractions were prepared from healthy MCF-7 cells or MCF-7 cells treated for 6 hr with staurosporine, doxorubucin, or TNFα plus cycloheximide. Proteins were resolved by gel electrophoresis, and the blot was probed with antibody to Bim.

(C) Mitochondrial and nuclear lysates were prepared from healthy MCF-7 cells or MCF-7 cells 6 hr after UV irradiation. Lysates were immunoprecipitated with antibody to Bcl-2. Anti-Bcl-2 immunoprecipitates were resolved on a gel, and the blot was probed with antibodies to Bcl-2 (top panel), Bim (middle panel), or LC8 (lower panel). Asterisk, IgL chain from the antibody used for IP.

MCF-7 cells by treatment with UV irradiation, staurosporine, doxorubucin, or TNFα plus cycloheximide (Huang t al., 1997). To determine the subc llular localization f Bim$_L$ and other proteins in these cells, extracts were treated with taxol to stabilize the microtubules and then solubilized with Triton X-100. In healthy cells, most Bim$_L$ (~80%–90%) was present in the insoluble fractions. All of Bim$_L$ was released from the insoluble fraction within 6 hr of UV irradiation, whereas α-tubulin remained in the insoluble fraction. Bcl-2 was exclusively found in the soluble fraction of both healthy cells and cells undergoing apoptosis (Figure 6A). Relocalization of Bim$_L$ preceded detectable markers of apoptosis by several hours, and consistent with the results from FDC-P1 cells, the broad spectrum caspase inhibitor zVAD-fmk inhibited apoptosis but did not affect relocalization of Bim$_L$ and LC8. Treatment with staurosporine, doxorubucin, and taxol, but not with TNFα plus cycloheximide, also resulted in a shift in Bim$_L$ and LC8 localization (Figure 6B). This is consistent with the notion that UV radiation– and drug-induced apoptosis occurs by a mechanism that is controlled by the Bcl-2 family whereas "death receptor" signaling triggers apoptosis by a different

mechanism (Vanhaesebroeck et al., 1993; Strasser et al., 1995; Huang et al., 1997).

BH3-only proteins like Bim are thought to exert their proapoptotic activity by binding to Bcl-2 or its homologs and thereby blocking their antiapoptotic function (Adams and Cory, 1998). Coimmunoprecipitation experiments were performed to verify changes in subcellular localization of Bim_L and LC8 during induction of apoptosis by a second method. After UV irradiation, the amount of Bcl-2 associated with mitochondrial and nuclear fractions was unchanged. However, the amount of Bim_L and LC8 that could be coimmunoprecipitated with Bcl-2 from these organelles increased greatly after UV irradiation (Figure 6C). The relative abundance of Bim_L and LC8 indicates that most complexes containing Bcl-2 and Bim_L also contain LC8. Similar results were obtained when cells were treated with doxorubicin or staurosporine (data not shown). These results demonstrate that during induction of apoptosis, Bim_L and LC8 are released together from cytoskeletal structures and translocated to cytoplasmic membranes where they bind to Bcl-2.

Discussion

BH3-only proteins are potent inducers of apoptosis, and some appear to play a critical role in physiological cell death. This is exemplified by the finding that in *C. elegans*, deletion of the BH3-only protein EGL-1 results in a phenotype that is very similar to that caused by loss of the caspase CED-3 or its activator, CED-4: persistent survival of all somatic cells that normally undergo developmentally programmed cell death (Conradt and Horvitz, 1998). Since other cell death regulators are evolutionarily conserved between nematodes and man (Vaux et al., 1992; Hengartner and Horvitz, 1994), mammalian BH3-only proteins may also play an essential role in developmental cell death. Indeed, we have found that lymphocytes from Bim-deficient mice are resistant to certain apoptotic stimuli (P. Bouillet, A. S., S. Cory, and J. M. Adams, unpublished data).

Here we demonstrate that the proapoptotic activity of Bim is controlled by binding to LC8, which sequesters it to cytoskeleton-associated motor complexes, away from antiapoptotic Bcl-2 family members. This study documents regulation of a Bcl-2 family member during signaling for apoptosis using experimental systems in which cells express the proteins at physiological levels. Interpretation of previous studies on interactions of Bcl-2-related proteins has the caveat that they were carried out exclusively in overexpression systems. The interaction between Bim and the dynein motor complex was shown to have functional significance. Bim_S and Bim_L mutants that cannot bind to LC8 are more potent inducers of apoptosis than Bim_L or Bim_{EL} (Figures 3B and 3C), demonstrating that LC8-mediated coupling to the dynein motor complex regulates the cytotoxic activity of Bim. Although mRNA for *bim*$_S$ has been found (Hsu et al., 1998; O'Connor et al., 1998), we have not detected Bim_S protein in any of the tissues or cell lines surveyed to date (L. A. O., D. C. S. H., and A. S., unpublished data). Therefore, Bim_S may not be expressed normally, or its expression may be induced only in situations when a cell needs to be killed very rapidly. Since both physiologically expressed isoforms, Bim_L and Bim_{EL}, bind to LC8, it appears that in most cell types all Bim proapoptotic activity is controlled by sequestration to motor complexes.

It is informative to compare this regulatory mechanism with the posttranslational control of two other BH3-only proteins, Bad and Bid. The cytotoxic activity of Bad can be modulated by serine phosphorylation-dependent binding to 14-3-3 proteins. This interaction sequesters Bad away from sites where Bcl-2 and its functional homologs reside (Zha et al., 1996). When Bad is dephosphorylated at a critical serine residue, it translocates from 14-3-3 to Bcl-2 and Bcl-x_L and initiates the death program by neutralizing their antiapoptotic activity. Bad can be phosphorylated by the PI-3K/AKT pathway, which is activated by certain growth factor receptors (Datta et al., 1997; del Peso et al., 1997). It is therefore possible that Bad plays a critical role in developmental cell deaths that are initiated by limiting availability of cytokines.

Another BH3-only protein, Bid, is found as a 26 kDa polypeptide in the cytosol, where it is sequestered from antiapoptotic members of the Bcl-2 family (Wang et al., 1996). When caspase-8 is activated, it cleaves Bid and releases fragments of ~15 kDa and ~11 kDa. The ~15 kDa polypeptide relocalizes to mitochondria, where it binds to and antagonizes antiapoptotic members of the Bcl-2 family. This Bid fragment also promotes release of cytochrome c, which together with Apaf-1, dATP, and caspase-9 activates procaspase-3 (Li et al., 1998; Luo et al., 1998). If this is the sole mechanism for activating Bid, then it does not act as an initiator of apoptosis but rather appears to be involved in amplifying the process downstream of "initiator caspases" (Hengartner, 1998).

The control mechanism of Bim is clearly different from that of Bid and Bad. In contrast to Bid, relocalization and activation of Bim occurs independently of caspase activation, and we have not detected any Bim cleavage during apoptosis (Figures 5A, 5B, and 6). Unlike Bad, which dissociates alone from 14-3-3 during induction of apoptosis, Bim_L and LC8 are both released from the dynein motor complex and translocate together to Bcl-2 (Figures 5 and 6). Consistent with the notion that Bim_L/Bim_{EL} interact simultaneously with LC8 and Bcl-2, Bim_L/Bim_{EL} have separate binding sites for these two proteins (Figure 2). We are currently investigating the signaling mechanisms that regulate binding of Bim-LC8 to the dynein motor complex and how this interaction is disrupted during the initiation of apoptosis. Bim does not regulate the association between LC8 and the dynein motor complex since LC8 is released in IL-3-deprived FDC-P1 cells, which do not express Bim (Figure 5C). Additional posttranslational modifications, apart from binding to LC8, may influence subcellular localization and activity of Bim. For instance, Bim_L and Bim_{EL} bind LC8 with apparently similar affinity, but Bim_{EL} is a less potent killer than Bim_L (O'Connor et al., 1998), indicating that there may be additional regulatory mechanisms.

The model we propose from these studies is one in which BH3-only proteins act as sensors of vital cellular

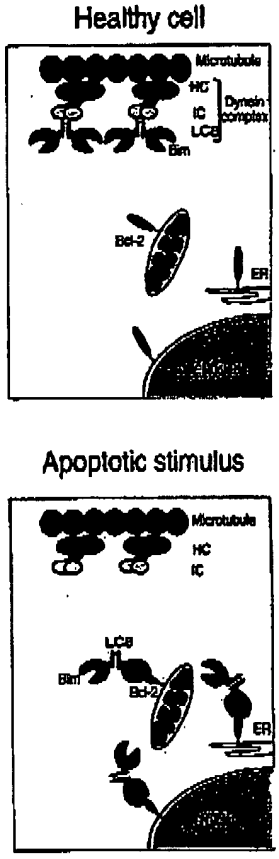

Figure 7. Model for the Regulation of Bim

In healthy cells, Bim_L is bound to LC8 and thereby sequestered to the microtubule-associated dynein motor complex, which also contains dynein intermediate chains (IC) and dynein heavy chains (HC). Certain apoptotic stimuli cause release of a complex of Bim_L and LC8. Free Bim_L, still complexed with LC8, binds to Bcl-2 or its functional homologs and thereby neutralizes their ability to block adaptor protein-mediated activation of initiator caspases.

processes (Figure 7). In the case of Bim_L and Bim_{EL}, they act in concert with their binding partner, LC8, to monitor processes on the cytoskeleton. When vital cellular processes, such as cell adhesion, intracellular transport, or stimulation by growth factors are disturbed, BH3-only proteins are unleashed and act as death ligands that counteract the antiapoptotic activity of Bcl-2-like proteins.

A more complete understanding of cell death control will require knowledge of the processes that regulate the activity of the other BH3-only proteins. Protein interaction screens and biochemical experiments with mammalian Blk/Nbk, Harakiri/DP5, and Blk, and *C. elegans* EGL-1 may reveal novel mechanisms for apoptosis induction. Since mammals have at least six BH3-only proteins, it is likely that some degree of functional overlap exists. It will be interesting to see whether in a particular cell type distinct death signals are transmitted via activation of different BH3-only proteins or whether several of these proteins become activated. We speculate that Bim_L and Bim_{EL} play a critical role in those apoptotic signals that impinge on the cytoskeleton and motor complexes, for instance, when adherent cells lose their contact with the extracellular matrix, a process called "anoikis" (Ruoslahti and Reed, 1994). It is interesting to note that Bim_L and Bim_{EL} are expressed at relatively high levels in many types of epithelial cells (Figures 1F and 6). Detailed analysis of the expression patterns of the various BH3-only proteins and generation of mutant mice lacking one or several of these proteins are expected to answer these questions and will increase understanding of apoptosis and tissue development.

Experimental Procedures

Yeast Two-Hybrid and Reverse Yeast Two-Hybrid Screening

Saccharomyces cerevisiae strain HF7c (Clontech) was used for two-hybrid screening. Transformations were carried out as described (Gietz and Woods, 1994). Around 4×10^8 clones were screened on −H (histidine), −L (leucine), and −W (tryptophan) plates. Clones that grew after incubating at 30°C for 5 days were assayed for β-galactosidase activity (Duttweiler, 1996). Plasmid rescue was performed as described (Wach et al., 1994).

The reverse yeast two-hybrid screen was performed according to a published protocol (Vidal et al., 1996). The target gene, *bim_L*, was mutagenized by low-fidelity PCR. Template DNA (100 ng) was amplified in 100 μL reaction buffer (50 mM KCl, 10 mM Tris [pH 9.0], 0.1% Triton X-100, 1.5 mM $MgCl_2$, 1 μg/μL BSA, and 5 U of Taq DNA polymerase (GIBCO–BRL) using 0.2 μM of each primer. Primers used were: sense, 5′-AGGATCCACCATGGCCAAGCAACC; and antisense, 5′-CCGGGCGCAGATCTTCAG. The concentrations of the nucleotides were independently modified. In each reaction, a particular nucleotide was limiting at 20 μM, while the others were kept at 100 μM. After 10 cycles, 100 mM $MnCl_2$ was added, and reactions continued for another 30 cycles. Pooled PCR products, linearized *bim_L* vector in pGAD424, and the binding partner, pGBT9 LC8, were cotransformed into *S. cerevisiae* strain MAV 103. Transformants were replica plated onto medium containing 0.2% 5-FOA (Diagnostic Chemical Co.), replica cleaned twice, and incubated for a further 3 days. Resistant clones were screened for production of full-length Bim_L by curing the clones of the LC8 vector and then mating with strain MAV 203 transformed with pGBT9 Bcl-2. Since the BH3 domain in Bim_L is C-terminal relative to the region subjected to mutagenesis, Bim_L clones harboring nonsense mutations would not interact with Bcl-2 in this assay. The remaining clones were rescued and sequenced.

Expression Vectors and Site-Directed Mutagenesis

EE epitope-tagged expression vectors, EE-Bim_S, EE-Bim_L, EE-Bim_{EL}, EE-Bak, EE-Bax, EE-Bad, EE-Blk, EE-Bid, and Bcl-2, and Bcl-x_L expression vectors have been described (Huang et al., 1997; O'Connor et al., 1998). Expression constructs encoding Bim_L mutants were generated by subcloning or by PCR using proofreading *Pfu* DNA polymerase (Stratagene). Products were subcloned into pEF EE pGKhygro, which incorporates an N-terminal EE epitope tag (Grussenmeyer et al., 1985). The LC8 cDNA was cloned into pEF FLAG pGKpuro (Huang et al., 1997). Truncated mutants of LC8 were generated by PCR using *Pfu* DNA polymerase and were cloned into pEF FLAG pGKpuro.

The bait for yeast two-hybrid screening was generated by cloning a cDNA encoding amino acids 1–122 of mouse Bim_L into pGBT9 (Chien et al., 1991) (Clontech). For reverse two-hybrid screening, a cDNA encoding LC8 was cloned into pGBT9, and a cDNA encoding amino acids 1–122 of mouse Bim_L was inserted into pGAD424 (Chien et al., 1991) (Clontech). A control construct, pGBT9 Bcl-2, was generated by subcloning a cDNA encoding amino acids 1–217 of human Bcl-2 into pGBT9. All constructs were verified by automated sequencing (ABI Perkin Elmer) using PRISM or BIGDYE terminator. Details of oligonucleotides used for generating constructs will be supplied on request.

Cell Culture, Transfections, and Cell Death Assays

Cell lines used were: 293 T, a human embryonic kidney line; MCF-7, a human breast carcinoma line; FDC-P1, a mouse IL-3-dependent promyelocytic line, and L929, a mouse fibroblastoid line. Culture conditions for these lines have been described (Huang et al., 1997). 293 T cells were used for liposome-mediated transient transfection (GIBCO-BRL). FDC-P1 cells stably expressing Bcl-2 and EE-tagged Bim isoforms or Bim_L mutants have been described (O'Connor et al., 1998) or were generated by electroporation and selection in 4 μg/mL puromycin (Sigma) and/or 2 mg/mL hygromycin (Boehringer Mannheim). Clones expressing high levels of the protein of interest were selected by immunofluorescence staining of fixed and permeabilized cells and flow cytometric analysis on a FACScan (Becton Dickinson) (Strasser et al., 1995). Colony formation of transfected L929 cells was assayed as described (O'Connor et al., 1998).

Apoptosis was induced in FDC-P1 cells by IL-3 deprivation or by 10 Gy γ radiation. MCF-7 cells were treated with UV irradiation at a dose of 100 J/m², 1 μM staurosporine (Sigma), 500 ng/mL doxorubucin (David Bull Laboratories), 10 nM taxol (Sigma), or 1 μg/mL human TNFα (gift of G. Wong) and 25 μg/mL cycloheximide (Sigma). The broad spectrum caspase inhibitor zVAD-fmk (Bachem) was used at 50 μM. Cell survival was quantitated by flow cytometric analysis of cells stained with 5 μg/mL propidium iodide (Sigma).

Protein Extraction, Immunoprecipitation, Immunoblotting, Velocity Sedimentation on Sucrose Gradients, and Subcellular Fractionation

Cell lysates were prepared in lysis buffer (20 mM Tris–HCl [pH 7.4], 135 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X–100, 10% glycerol) supplemented with 0.5 μg/mL Pefabloc and 1 μg/mL for each of leupeptin, aprotinin, soya bean trypsin inhibitor, and pepstatin (Sigma). For immunoprecipitations, lysates were centrifuged at 900 g for 5 min to remove nuclei and debris prior to precleaning with protein G Sepharose (Pharmacia). Lysates were incubated with 1–3 μg of monoclonal antibodies: mouse anti-*EE* (BabCO), mouse anti-FLAG M2 (Sigma), mouse anti-human Bcl-2 (Bcl-2-100) (Pezzella et al., 1990), rat anti-Bim_L (clone 5E5) (O'Reilly et al., 1998), or rabbit antibodies to Bcl-x (Pharmingen). Immune complexes were captured with protein G Sepharose. Beads were washed 3–6 times in lysis buffer and boiled in SDS sample buffer. Proteins were size fractionated on SDS–PAGE gels (Novex) and transferred onto nitrocellulose membranes (Amersham). Filters were probed with the antibodies described above or with affinity-purified rabbit antibodies raised against *Chlamydomonas* LC8 (King et al., 1996), dynein intermediate chain, and α-tubulin (Sigma). Bound antibodies were revealed with HRP-conjugated antibodies: sheep anti-mouse Ig, sheep anti-rabbit Ig, or goat anti-rat Ig (AMRAD Biotech) followed by enhanced chemiluminescence (Amersham).

Metabolic labeling of 293 T cells was done with 100–200 μCi/mL of [^{35}S]methionine (NEN) in methionine-free medium (GIBCO–BRL). In FDC-P1 cells, metabolic labeling was done with 10 μCi/mL of [^{35}S]methionine in DMEM containing 10% fetal calf serum plus IL-3. Equivalent amounts of trichloroacetic acid (Sigma) precipitable counts (10^6–10^7 cpm) were taken for each immunoprecipitation.

For velocity sedimentation (Tatu et al., 1995), FDC-P1 cells were treated with 5 μM taxol (Sigma) for 4 hr and 0.5 mM AMP-PNP for 30 min before lysis. Cleared lysates were loaded on a 5–20% sucrose gradient in 20 mM MES, 100 mM NaCl, 30 mM Tris–HCl (pH 7.0), and 0.1% Triton X-100. Centrifugation was done in a SW41 Ti rotor (Beckman) at 40,000 rpm for 18 hr and fractions were collected manually. For separation of the microtubule fraction from the Triton X-100 soluble fraction (Solomon, 1986), cleared lysates were treated with taxol (5 μg/10^6 cells in 1 mL) and apyrase (Sigma) 2U/mL for 15 min at 30°C. The lysate (400 μL) was loaded on 1 mL of a 20% sucrose cushion and spun at 100,000 rpm in a TLA45 rotor (Beckman).

Nuclei and mitochondria were purified according to a published protocol (Hagenbüchle and Wellauer, 1992). At least 3 × 10^9 cells were homogenized in a dounce homogenizer in 5 mL mitochondrial medium (20 mM HEPES [pH 7.5], 0.3 M sucrose, 1 mM EDTA plus protease inhibitors). Nuclei and debris were removed by centrifugation at 700 g for 10 min (SS34 rotor, Sorvall). Pellets were saved for purifying nuclei. To the supernatant, 15 mL of mitochondrial medium was added and centrifuged at 6000 g for 10 min (SS34 rotor Sorvall). The pellet was washed in 20 mL of mitochondrial medium, resuspended in 3 mL, and layered on a gradient of 3 mL each of 1.2 M, 1.3 M, and 1.6 M sucrose dissolved in mitochondrial medium and centrifuged at 100,000 g for 1 hr at 4°C (SWTi45 rotor Beckman). The mitochondrial band at the 1.3 and 1.6 M sucrose interphase was isolated. Triton X-100 was added to a final concentration of 1%.

Pellets containing nuclei and debris were resuspended in 10 mL mitochondrial medium and layered on a 10 mL cushion of 0.9 M sucrose in buffer A (60 mM KCl, 15 mM NaCl, 15 mM HEPES [pH 7.8], 14 mM 2-ME plus protease inhibitors) and centrifuged for 10 min at 3500 rpm at 4°C in a Sorvall HB-4 rotor. The pellet was resuspended in 10 mL buffer A and sedimentation on a sucrose cushion repeated. The final pellet was resuspended in 1 mL of buffer B (75 mM NaCl, 0.5 mM EDTA, 20 mM Tris–HCl [pH 7.9], 0.8 mM DTT, 50% glycerol plus protease inhibitors). Before immunoprecipitation, nuclei were dissolved in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS).

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